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Juvenile Idiopathic Arthritis

Modulation of Warfarin Sodium

Highlights

The Genomics of Liposarcoma

Genetic Manipulation of Dormancy

Discovering Thoughts, Inventing Future

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Contents of the Issue

i. Copyright Notice
ii. Editorial Board Members
iii. Chief Author and Dean
iv. Contents of the Issue

1. Modulation of Warfarin Sodium into Warfarin Potassium for Patients with Hypertension. *1-14*
2. The Genomics of Liposarcoma: A Review and Commentary. *15-33*
3. Epigenetics Theoretical Limits of Synthetic Genomes: The Cases of Artificials *Caulobacter* (*C. eth-2.0), Mycoplasma Mycoides (JCVI-Syn 1.0, JCVI-Syn 3.0 and JCVI_3A), E-coli and YEAST chr XII*. *35-47*
4. Physiological and Molecular basis of Dormancy in Yam Tuber: A Way Forward towards Genetic Manipulation of Dormancy in Yam Tubers. *49-75*
5. Physical and Mechanical Properties of Teak (Tectona Grandis L.Fil.) Thermo-Plywood from Plantations in Lao P.D.R. *77-84*
6. Auto Inflammation Candidate Genes in Juvenile Idiopathic Arthritis. *85-93*
7. Petrological, Geochemical and Mechanical Characteristics of Kooz Kunarr Khewa Calcite Marble. *95-103*
8. Antimeiotic Properties of the Aqueous Extracts of Leaves, Fruits and Roots of the Muskmelon *Cucumis Melo* L. (Cucurbitaceae) in the Pest Grasshopper *Zonocerus Variegatus* L. (Pyrgomorphidae). *105-111*
9. Cross - Century Discovery: Mendelian Dualistic Genetics. *113-117*

v. Fellows
vi. Auxiliary Memberships
vii. Preferred Author Guidelines
viii. Index

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Modulation of Warfarin Sodium into Warfarin Potassium for Patients with Hypertension

By Al-Baraa Akram
De Montfort University

Abstract- Warfarin is an oral anticoagulant drug that has a prolonged duration of action with delayed onset of action; its chemical structure contains sodium atoms, which may be hazardous to hypertensive patients. To solve this problem, sodium atom can be substituted with potassium or lithium atom which can help the patient to relieve hypertension in addition to it essential role as an anticoagulant.

Another advantage of this preparation is that it can be used as an antidote against digitalis toxicity, but the warfarin interactions with other drugs are still the same as a cytochrome P450 inhibitor.

*Keywords: warfain, hypertension, international normalized ratio, therapeutic window, clinical trials, pharmaco-genomics, CYP2C9*3.*

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Modulation of Warfarin Sodium into Warfarin Potassium for Patients with Hypertension

Al-Baraa Akram

Abstract- Warfarin is an oral anticoagulant drug that has a prolonged duration of action with delayed onset of action; its chemical structure contains sodium atoms, which may be hazardous to hypertensive patients. To solve this problem, sodium atom can be substituted with potassium or lithium atom which can help the patient to relieve hypertension in addition to it essential role as an anticoagulant.

Another advantage of this preparation is that it can be used as an antidote against digitalis toxicity, but the warfarin interactions with other drugs are still the same as a cytochrome P450 inhibitor.

Warfarin reduces blood clotting by inactivating vitamin K epoxide reductase, which activates vitamin K1, the main component in the blood clotting process. Without sufficient vitamin K1 activation, clotting factors II, VII, IX and X have decreased clotting ability. The anticlotting protein C and protein S have also inhibited, but to a lesser degree. A few timesare required for the clotting process, and these effects can take about five days. Additionally, because this process requires enzymes like VKORC1, patients who take warfarin with polymorphism of these enzymes can require adjustment as genetic factors should be taken into consideration, thus may require lower doses.

*Keywords: warfain, hypertension, international normalized ratio, therapeutic window, clinical trials, pharmaco-genomics, CYP2C9*3.*

I. Introduction

a) History and overview

The history of warfarin discovery started in the 1920s in the prairies of North America and Canada. Cattle were dying from internal bleeding without any precipitating cause, which led to a dietary query problem that many farmers complained. They called it sweet clover disease, and at this time, they recommended each other not to feed their cattle the moldy sweet clover hay.

But science had a different opinion, a research work funded by the Wisconsin Alumni Research foundation patented in 1941. Variation of dicoumarol was patented as a rat poison in 1948 and then transitioned to the clinical application under the name of Coumadin.

The prefix of the name warfarin was derived from WARF (the first letters of Wisconsin Alumni Research Foundation), and the suffix –arin was derived from coumarin.

Warfarin first came for large-scale commercial use in 1948 as a rat poison (1). Warfarin was officially approved for human use by the United States food and drug administration (FDA) to treat blood clots in 1954 (2). In 1955 warfarin's reputation as a safe and acceptable treatment was bolstered when American president Dwight Eisenhower took warfarin because of a massive and publicized heart attack (3). This story kick-started the usage of warfarin in coronary heart disease, arterial plaques, and ischemic heart attacks. It is listed in the World Health Organization (WHO) as the essential medicine. Warfarin is available as a generic medication. In 2019 it was the 50th most prescribed medication in the United States, with more than 14 million prescriptions (4).

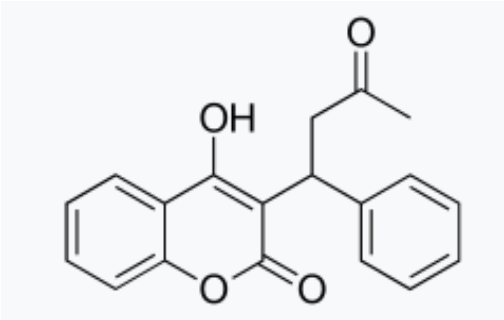

Figure 1: Chemical structure of warfarin with empirical formula $C_{19}H_{16}O_4$

b) Platelet response to vascular injury

Physical trauma to the vascular system e.g., punctures or cuts initiate a complex series of interactions between platelets, endothelial cells, and coagulation cascade. This results of formation a platelet-fibrin plug or clot at the site of puncture.

The creation of a thrombus involves many of the same steps as normal clot formation, except this trigger stimulation which is a pathological case in the vascular system.

c) Coagulation cascade (secondary hemostasis)

Series of protease enzymes and their cofactors takes place of phospholipids' surface, platelet, and endothelium which consist of extrinsic, intrinsic, and common pathways that results in the formation of stable fibrin clot as shown in figure 2.

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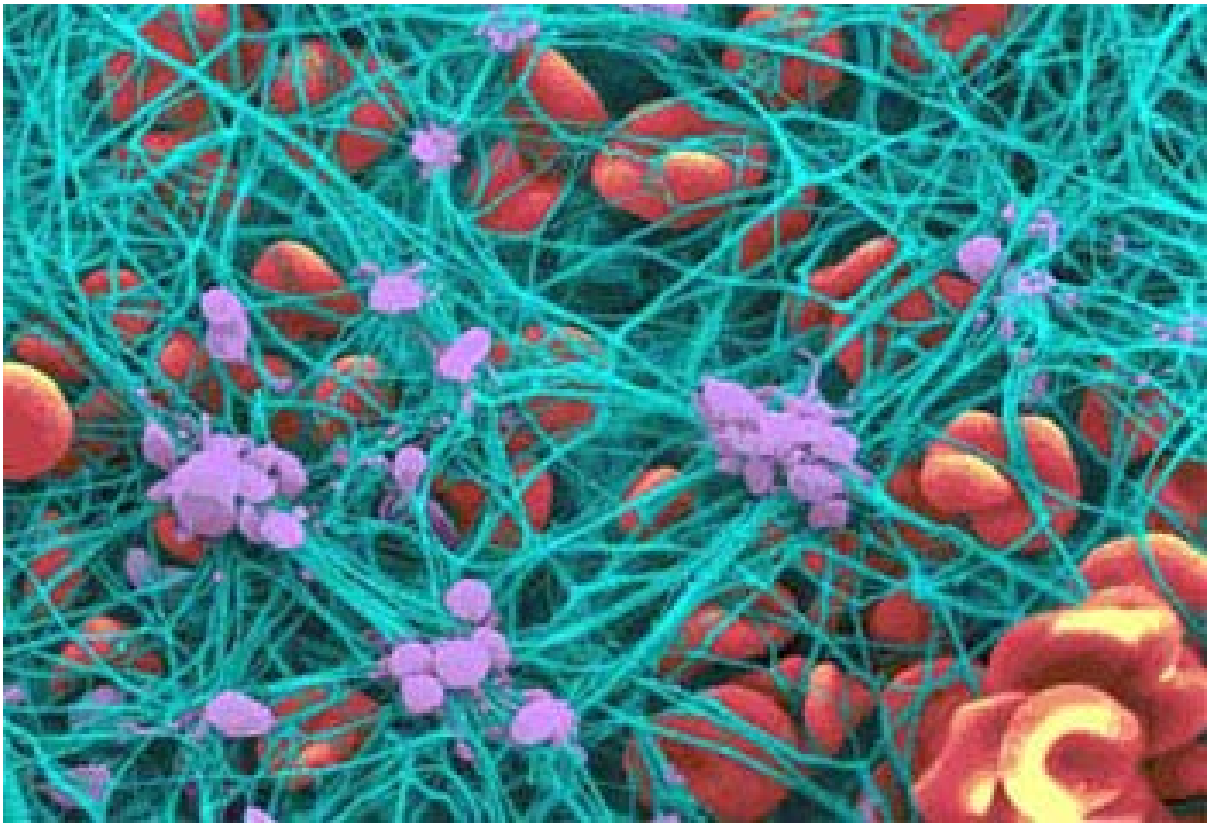

Figure 2: Phospholipids surface, platelet and endothelium, consist of extrinsic, intrinsic and common pathways results in formation of stable fibrin clot

i. *Resting platelets*

Platelets act as a vascular guard, monitoring the integrity of the endothelium; in the absence of injury, resting platelets circulate freely. Chemical mediators, such as prostacyclin and nitric oxide, are synthesized by intact endothelial cells and act as platelet aggregation inhibitors. Prostacyclin works by integration with platelet membrane receptors coupled to the synthesis of cAMP →increased cAMPis associated with the decrease of intracellular Ca^{+2}, which leads to inhibition of platelet aggregation.

Damaged endothelial cells synthesize lower prostacyclin; thus the binding of prostacyclin to platelet receptors is decreased, leading to lower levels of intracellular cAMP, which leads to platelet aggregation.

ii. *Platelet adhesion*

When the endothelium is injured, platelets adhere to and cover the exposed collagen of the sub-endothelium

iii. *Platelet activation*

Receptors on the surface of the adhering platelets are activated→morphologic changes in theplatelets→ release of platelet granules containing chemical mediators, such as adenosine diphosphate (ADP), thromboxane A2, serotonin, platelet activation factor and thrombin.

1. *Platelet aggregation*

The increase in the cytosolic Ca^{+2} accompanying activation leads to:

- The release of platelet granules containing mediators, such as ADP and serotonin, that activate other platelets
- Activation of thromboxane A2 synthesis
- Activation of glycoprotein GP;IIb/IIIa receptors that bind fibrinogen and ultimately regulate platelet-platelet interaction and thrombus formation

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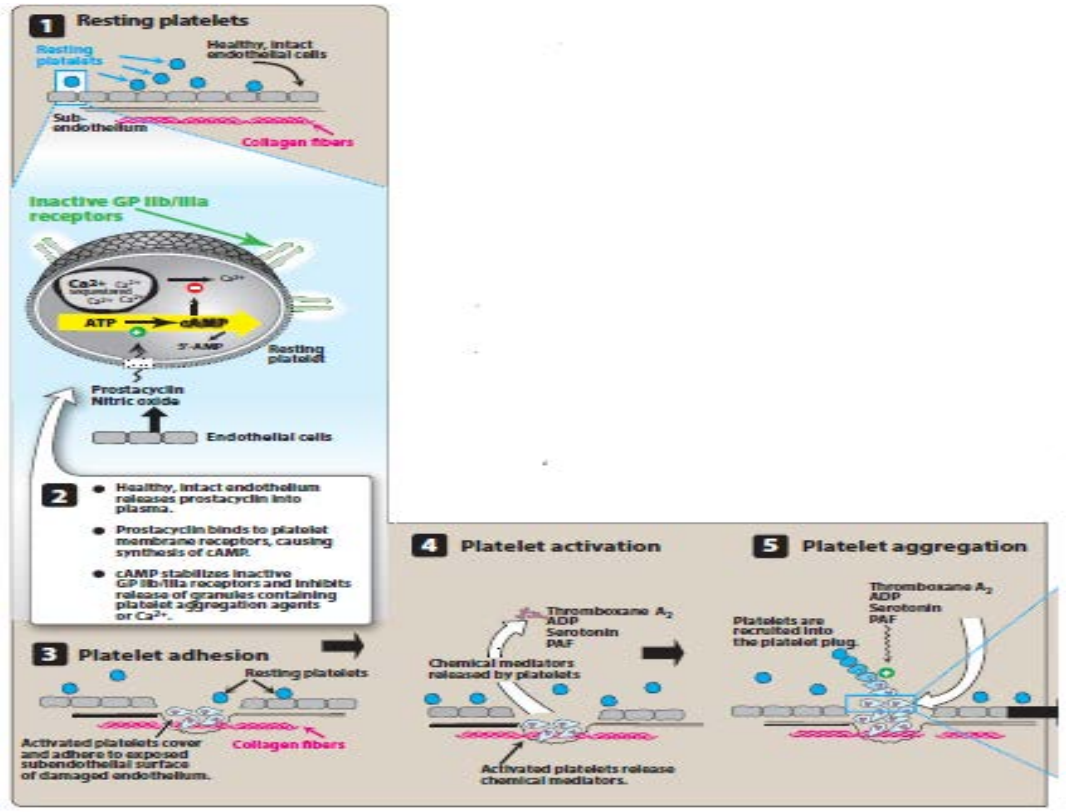

Figure 3: Scheme for Coagulation cascade (secondary hemostasis) showing the four phases of thrombus formation, which are resting platelets, platelet adhesion, platelet adhesion, and platelet aggregation

Fibrinogen which is a soluble plasma glycoprotein simultaneously binds glycoprotein IIb/IIIa receptors on two separate platelets, platelet cross-linking and platelet aggregation; this leads to a mass of platelet aggregation because each activated platelet can recruit other platelets.

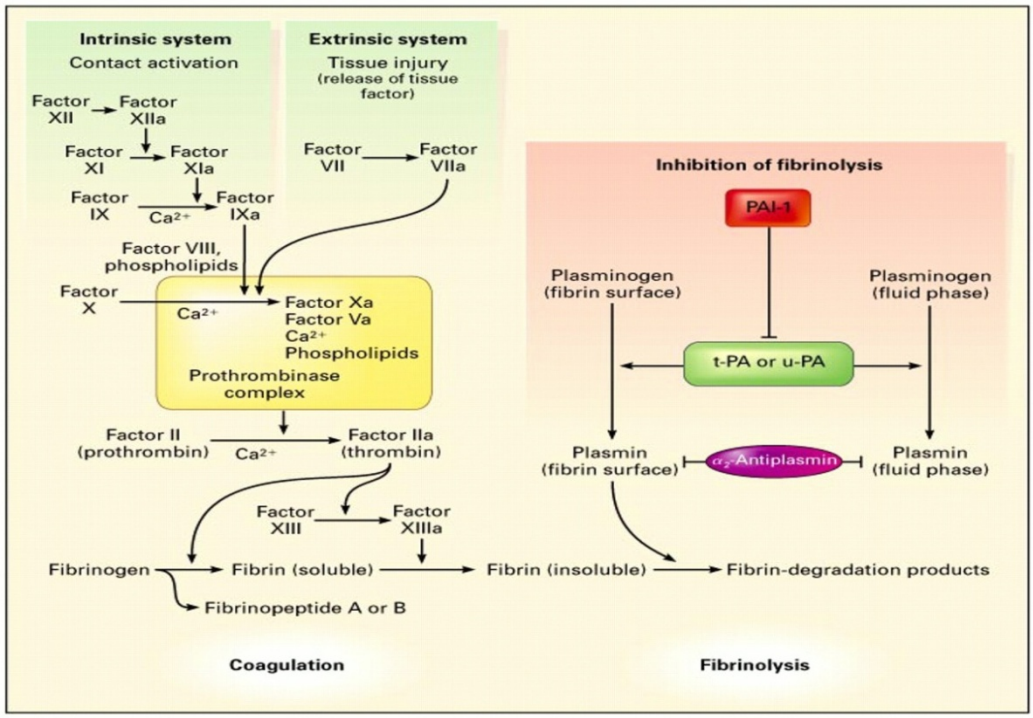

Figure 4: The Coagulation and Fibrinolytic Pathways in normal cells

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d) Medical Uses

Warfarin is used to treat the tendency of thrombosis or as secondary precaution, preventing further episodes in those individuals who have already formed a blood clot, thrombus; warfarin treatment can be used to prevent other formation of blood clots from reducing the risk of an embolism which defined as the migration of blood clot to block the blood supply of a vital organ (5).

Warfarin is best suited as an anticoagulant to inhibit clot formation in areas of slow running blood, such as the veins and pooled blood behind artificial and natural valves and in the pooled blood in dysfunctional heart atria parts. Thus, common clinical indications for warfarin use are atrial fibrillation, the presence of artificial heart valves, deep venous thrombosis, and pulmonary embolism, where the embolized clots form in the veins. Warfarin is used also as an antiphospholipid syndrome. It has been used occasionally after heart attacks or myocardial infarction, but it is less effective in treating new thrombus in coronary arteries.

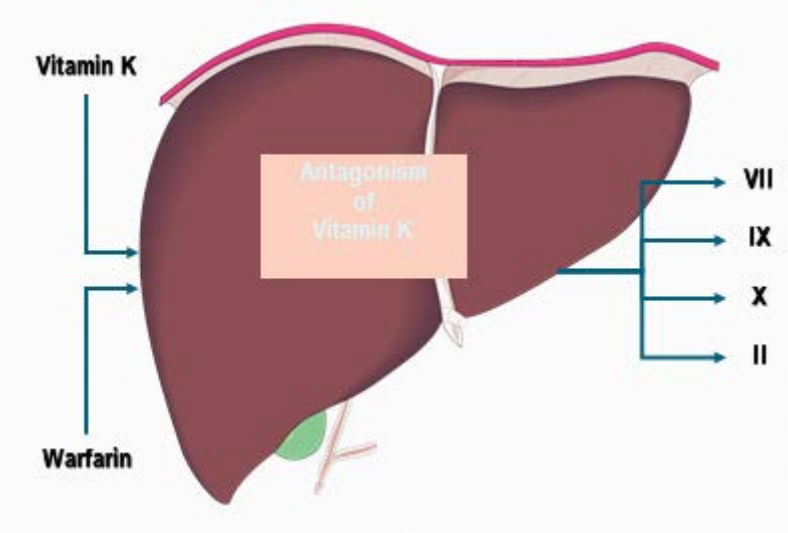

Figure 5: Warfarin and vitamin K mechanism of action showing antagonism of vitamin K synthesis in the liver of non-functional coagulation factors in the presence of vitamin K epoxide reductase

Prevention of clotting in arteries is usually undertaken using antiplatelet drugs, which work by a different mechanism from warfarin which does not affect the platelet function. It can be used to treat people from strokes result from a trial fibrillation, although direct anticoagulants which are taken orally may offer more significant benefits.

The maintenance dose of warfarin can be fluctuated according to the levels for vitamin K1in the diet. Keeping vitamin K1 intake at stable levels can reduce this fluctuation. Green leafy vegetables can be a good source for vitamin K1. Green parts of members of the family a piaceae such as parsley, cilantro and dill are rich sources of high levels of vitamin K1; cruciferous vegetables as cabbage and broccoli as well as lettuces dark green parts also participate in obtaining vitamin K. Green vegetables such as green bean don't have the same amounts of vitamin K1 like green leafy vegetables. Specific vegetable oils have certain parts of vitamin K1. Foods low in vitamin K1 like roots, bulbs, tubers, and most fruit juices. Cereals, grains, and other milled products are also poor with vitamin K1 (6).

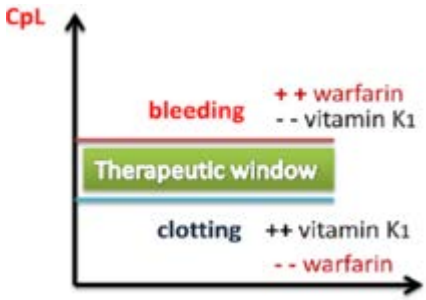

Figure 6: Vitamin K1-warfarin interaction effect with an inverse proportion between warfarin intake and vitamin K1. When warfarin levels are increased, people have more subjected to bleeding. Conversely, lower levels of warfarin lead to high risk of clotting. There is a narrow range when the benefits of warfarin are more significant than the risks, its therapeutic window as well as there are some interactions between some foods and warfarin intake

e) Self-testing

Anticoagulation of warfarin can be measured at home. There is a narrow range when the benefits with warfarin are greater than the risks, its therapeutic window as well as there are some interactions between some foods and warfarin intake, currently available self-

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management devices give accurate international normalized ratio (INR) results comparable with average results obtained from laboratories (7).

f) Alternative anticoagulants

In some countries, other coumarins are used instead of warfarin, such as phenprocoumon. These drugs have a shorter (acenocoumarol) or longer (phenprocoumon) half-life, and are not wholly interchanged with warfarin. Several types of these drugs can offer the efficacy of warfarin without needing monitoring, such as dabigatran, apixaban, edoxaban, and rivaroxaban; they have been approved rather than classical warfarin. Complementing these drugs are available for dabigratan, apixabanan drivaroxaban which is suggested for edoxaban, but its use is considered off label because of limited evidence. A reversal agent for dabigratan, apixaban, edoxaban and rivaroxaban is in development (8).

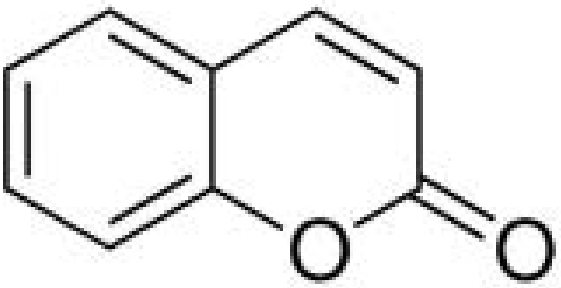

Figure 7: Chemical structure of coumarin molecule. Its molecular weight of 146.1427 g/mol, a boiling point equals 301.7 degrees Celsius, with a density of 935 kg/m^3 and a melting point of 71 degrees Celsius.

g) Contraindications of warfarin during pregnancy

Warfarin is contraindicated during pregnancy, as it passes through the placental barrier and can cause severe bleeding to the fetus; warfarin use in pregnancy is usually associated with spontaneous abortion, stillbirth, neonatal death, and preterm birth. Coumarins such as warfarin are also teratogens; they cause congenital disabilities; the incidence of congenital disability among fetuses has a possibility of 5%, although this possibility can reach 30% in some studies depending on when exposure occurs during pregnancy, two distinct abnormalities can appear.

When warfarin, or any 4-hydroxycoumarin derivative, is given during the first trimester, particularly between the 6th and 9th week of pregnancy, a constellation of congenital disabilities is known as fetal warfarin syndrome, warfarin embryopathy can occur. Fetal warfarin syndrome is mainly characterized by skeletal abnormalities, which include nasal hypoplasia, a depressed or narrowed nasal bridge, scoliosis and calcifcation in the vertebral column, femur and heel bone which is shown in the X-rays. Limb abnormalities such as brachydactyly, usually short fingers and toes or underdeveloped extremities can also occur (9).Standard non-skeletal features of fetal warfarin syndrome include low birth weight and disabilities.

h) Warfarin adverse effects

1. bleeding

It is the most common side effect of warfarin. The risk of bleeding due to warfarin is low but definite; approximately the annual rate is 1% to 3%. And any benefits that need to outweigh the need to be considered. All types of bleeding are possible, but the most severe is spinal cord and brain bleeding, intracerebral hemorrhage, or hemorrhagic stroke (10).

The risk of bleeding can be increased if the international normalized ratio is out of range due to accidental overdose or drug interactions (11). This risk is highly increased if the international normalized ratio exceeds 4.5 (12).

Several risk scores appear with treatment using warfarin. A commonly used score is HAS-BLED includes known predictors of warfarin related bleeding, which are:

H=uncontrolled high blood pressure

A=abnormal kidney function

S=previous stroke

B=known bleeding condition

L=previous label international normalized ration while taking warfarin

E=elderly by defined age of 65 years or more

D=drug associated with bleeding or alcohol misuse

While their use is recommended in medical practice guidelines (13), they are good predictors of bleeding risk but are not efficient in detecting or even predict the risk of hemorrhagic stroke (14). Bleeding risk may become highly possible with people on hemodialysis (15). Another score used to assess bleeding risk with patients on anticoagulants, specifically with warfarin or Coumadin, is the ATRIA score, which uses a weighted additive score of clinical findings to detect bleeding danger (16). The risk of bleeding is increased when warfarin is taken with antiplatelet like clopidogrel, aspirin, or non-steroidal anti-inflammatory drug (NSAIDs).

i) Warfarin necrosis

It is rare, but it is a hazardous complication resulting from treatment with warfarin, which occurs shortly after commencing treatment with warfarin with patients who have a protein C deficiency. Protein C is a natural anticoagulant that, as most coagulation factors which are inhibited because of warfarin, requires vitamin K-dependent carboxylation for its activity. Because warfarin decreases protein C synthesis faster than the other coagulation factors, paradoxically, it can increase levels of coagulation when treatment is first begun, so most patients are given heparin to combat this problem

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which can lead to limb necrosis and even gangrene. Its natural counterpart, purpurafulminans, occurs among children homozygous of protein C mutations.

j) Osteoporosis

After initial reports which claim that warfarin reduces calcium bone, there is a link between warfarin use and osteoporosis-related fracture. For women taking warfarin for deep venous thrombosis, the risk of rib fractures and vertebral fractures is increased; other fracture types don't occur more commonly.

II. Materials and Methods

To manipulate the reaction of converting warfarin sodium into warfarin potassium, you need to ask some simple questions:

You have an organic compound (warfarin) $C_{19}H_{15}NaO_4$ with a molecular weight of 330.3 g/mol; it contains a sodium atom and must replace with an atom of potassium. The IUPAC name of warfarin sodium is sodium 2-oxo-3-(3-oxo-1-phenylbutyl)-2H-chromene-4-olate.

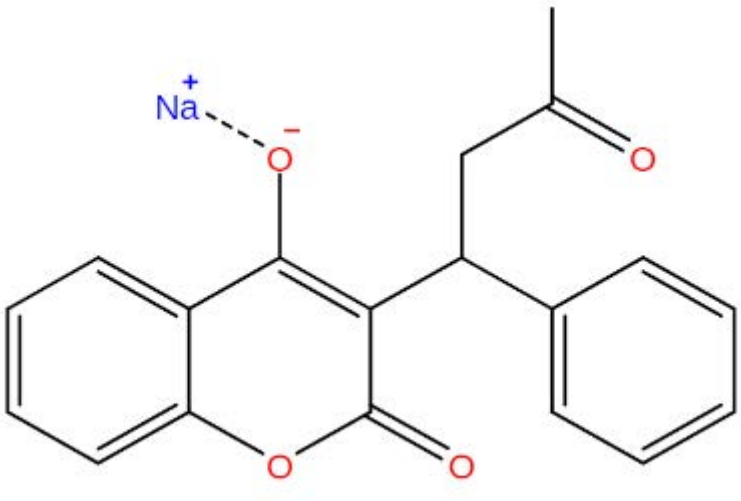

Figure 8: Chemical structure of warfarin sodium with a molecular formula $C_{19}H_{15}NaO_4$ and its molecular weight of 330.3 g/mol. It is a crystalline powder. Used as an anticoagulant and rodenticide

Which is better to replace the sodium atom with a potassium atom within this organic compound?

Is the simple substitution reaction preferred or the double substitution reaction?

1. If the simple substitution reaction was better, how can you get elementary potassium?
2. If the double replacement reaction is better, what is the concentration of potassium hydroxide required to complete the reaction? And is it better for substitution reactions or any potassium salt e.g., potassium iodide is better?

To answer these questions, you need to do the following:

Prepare an acid like hydrochloric acid to convert ONa group into OH, filter papers, beaker, conical flask, potassium base e.g., potassium hydroxide and mortar

1. In a mortar, grind 100 mg warfarin sodium tablets properly until you get the powder form to increase the surface area as shown in figure 9 below

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Figure 9: Grinding warfarin sodium tablets properly until you get the powder form to increase the surface area

2. Dissolve the powder of warfarin sodium in 100 ml water and add HCl to allow sodium to substitute hydrogen and convert the ON a group into OH in the warfarin molecule according to the chemical equation and as shown in figure 10

$$C_{19}H_{15}NaO_4+HCl+H_2O\rightarrow NaCl+ H_2O+C_{19}H_{16}O_4$$

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Figure 10: Dissolving the powder of warfarin sodium in 100 ml water and adding HCl to allow sodium to substitute hydrogen and converting the ON a group into OH in the warfarin molecule

3. Filter the solution in a filter paper, to get rid of the liquid and leave the powder to dry
4. Add the powder of warfarinback to fresh water and add potassium base as shown in figure 11 and chemical equation below

$$C_{19}H_{16}O_4 + KOH \rightarrow C_{19}H_{15}KO_4 + H_2O$$

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Figure 11: Adding the powder of warfarin back to fresh water and adding potassium base

5. Filter the solution again using a filter paper, then leave the powder to dry to get warfarin potassium, as shown in figure 12.

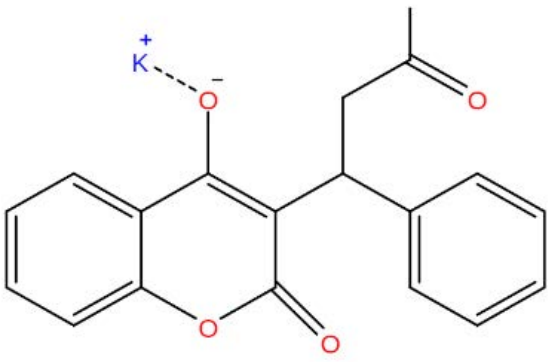

Figure 12: The chemical structure of warfarin potassium with a molecular formula $C_{19}H_{15}KO_4$ and its molecular weight of 319.4 g/mol, its IUPAC name is potassium 2-oxo-3-(3-oxo-1-phenylbutyl)-2H-chromene-4-olate

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N.B. After getting warfarin in the 3rdstep, you can use organic solvent to extract warfarin only then alkalize it using potassium base (potassium hydroxide); the OH group will be ionized into OK.

a) Spectroscopic assay

It is done by using nuclear magnetic resonance spectroscopy (NMR) to observe the local magnetic field around the atomic nuclei of the C13 atom. Warfarin potassium is placed in a magnetic field, and the signals are produced by exciting the nuclei sample of radio waves into NMR, which is detected with sensitive radio receivers. The intra-molecular area around the atom changes the frequency of resonance which gives access to the electronic structure of the molecule of warfarin potassium and its functional groups. Because the areas are like fingerprints as they are unique and very characteristic of each compound.

III. Results

Warfarin potassium powder has an increased surface area with good bioavailability. As an oral anticoagulant, this drug has a prolonged duration of action because potassium has a molecular weight more than sodium, so the diameter of the potassium atom is bigger and electron negativity is lower, leading to a delayed onset of action; its chemical structure contains potassium atom, which may be safe to the hypertensive patients. Sodium atom can be substituted with potassium or lithium atom, which can help the patient relieve hypertension in addition to its essential role as an anticoagulant.

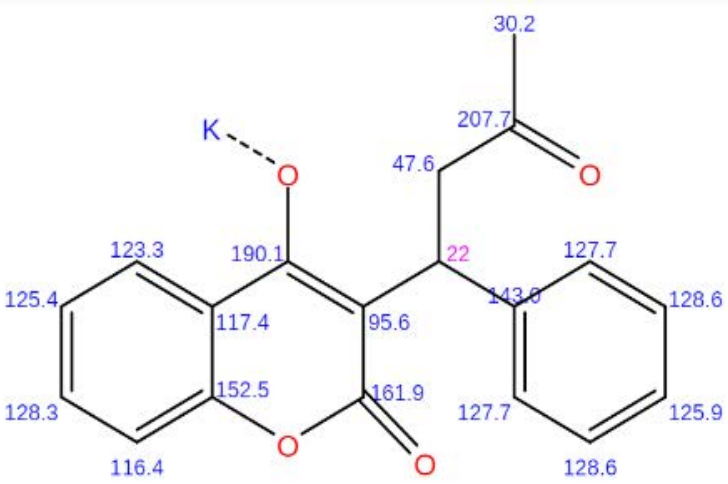

Figure 13: The chemical shift of warfarin potassium showing estimation quality of each atom indicated by colors; blue is good, pink is medium, and red is rough

a) Spectroscopic assay

In a simple term, the paramagnetic NMR spectroscopy of warfarin potassium whose IUPAC name is potassium 2-oxo-3-(3-oxo-1-phenylbutyl)-2H-chromene-4-olate, was given a chemical shift range which was spanned up to thousands of parts per million.

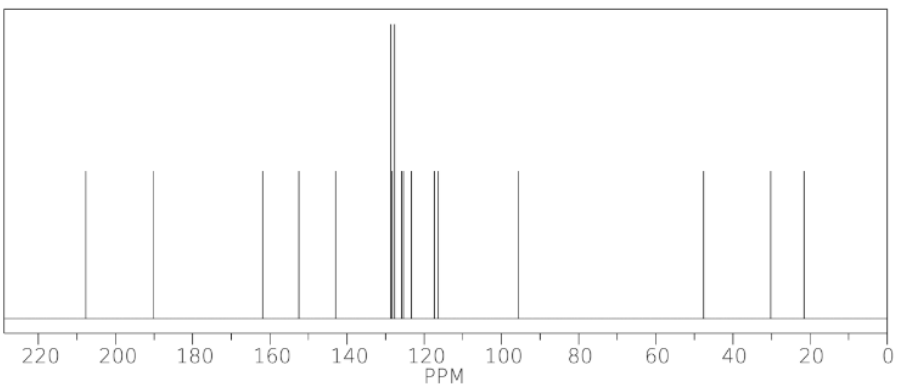

Figure 14: ^{1}H NMR spectroscopic assay of carbon atoms in warfarin potassium compound withchemical shift on the horizontal axis, each magnetically equivalent proton has a characteristic change and coupling to other protons appear as splitting of the peaks into multiplets between zero and 220 ppm

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b) Pharmacokinetics of warfarin potassium

Warfarin potassium is given orally, not parenteral; it is highly bound to plasma protein by 99% and metabolized extensively by the liver microsomal enzymes, so possible drug-drug interactions should be considered. Half-life is 40 hours, and its duration of action is 2 to 5 days.

It has an enterohepatic circulation, and its metabolites are excreted in urine and stool.

IV. Administration

The usual dose is 5 mg daily for 2 to 4 days, followed by maintenance of 2 to 10 mg daily as indicated by the measurement of the INR, which value derived from the ratio between patients' prothrombin time (PT) to reference prothrombin time.

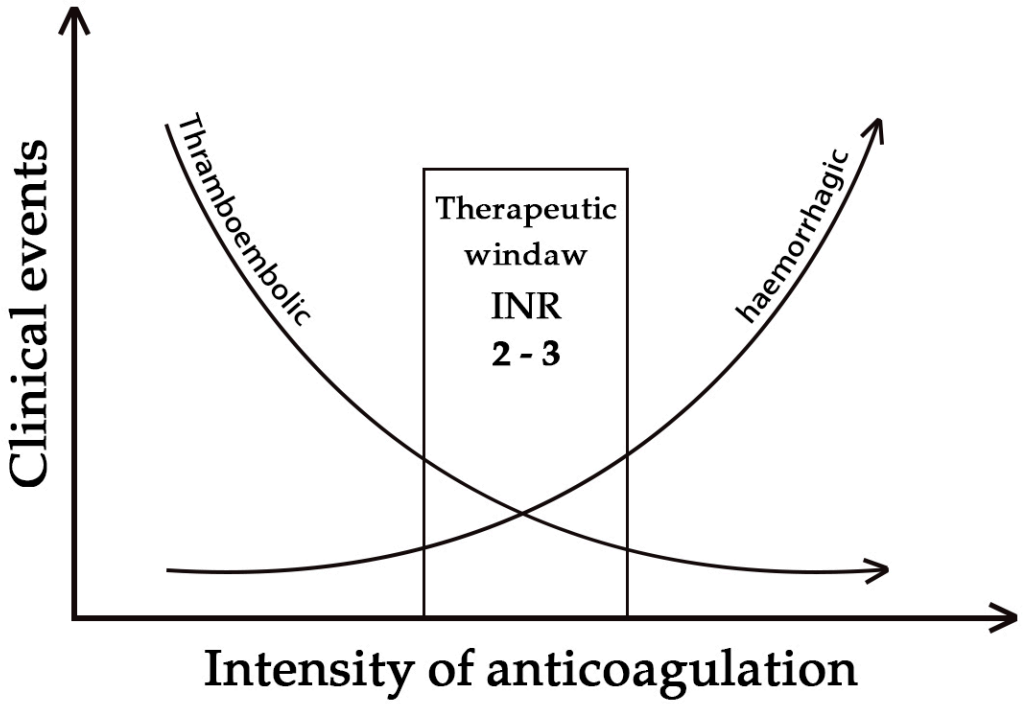

Figure 15: The intensity of warfarin potassium versus the clinical events to measure the therapeutic window and international normalized ratio of both thromboembolic and hemorrhagic state

a) Therapeutic uses

1. Prevention or prophylaxis against deep venous thrombosis (DVT) or pulmonary embolism
2. Prevention of systemic embolism in patients with acute myocardial infarction

The main side effects of warfarin potassium are bleeding, fetal malformation, and abortion if given during the pregnancy. So the antagonist of warfarin K overdose is vitamin K.

b) Factors influence warfarin potassium activity and its interactions

Warfarin interacts with many drugs because it is metabolized by the liver's microsomal enzymes; it can decrease the effectiveness of some drugs and increase the effectiveness of another

The reduced activity and energy, it affects the followings:

1. Reduced absorption because of malabsorption syndrome or cholestyramine administration
2. Hypoproteinemia, as in nephrotic syndrome, due to its low half-life
3. Increased secondary metabolism due to liver microsomal enzyme induction drugs such as rifampicin, barbiturates, and carbamazepine
4. Ingestion of a large amount of food containing vitamin K or supplements
5. Abnormal vitamin K epoxide reductase due to mutation of VKORC1 gene

The increased activity and effectiveness, it affects the followings:

1. Decreased metabolism by enzyme inhibitors like amiodarone, azole antifungals such as clotrimazole and fluconazole, isoniazid, and metronidazole
2. Displacement from protein binding by loop diuretics and valproic acid

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3. Vitamin K deficiency because of insufficient vitamin K diet or antibacterial therapy, which suppresses intestinal flora
4. Low concentrations of coagulation factors such as liver failure, heart failure, and the hypermetabolic state as in hyperthyroidism

Relative contraindications of warfarin potassium therapy

- Pregnancy
- Situations where the risk of bleeding is greater than the clinical benefits of therapy
- Uncontrolled alcohol and drug abuse
- Unsupervised dementia or psychosis
- Severe hepatic disease and gastrointestinal tract bleeding
- Sub-acute bacterial endocarditis
- Recent head trauma or recent major surgery

Thrombolytics promote the dissolution of thrombi in occluded blood vessels through a fibrinolytic effect. They are described mainly for acute myocardial infarction. Fibrinolytic drugs rapidly dissolve thrombi by catalyzing the plasmin formation from plasminogen, so they are plasminogen activators.

These drugs create a fibrinolytic state when administered intravenously; thus, they are both protective and curative. They are given as a bolus or by intravenous infusion. Examples of these drugs are streptokinase and urokinase which is a human enzyme synthesized by the kidney, which converts plasminogen into plasmin.

Table 1: Comparison between heparin and warfarin potassium showing their effectiveness, route of administration, the onset of action, duration of action, mechanism of action, antidote, lab control, and safety in pregnancy

Character	Heparin	Warfarin Potassium
Effectiveness	In vivo and in vitro	In vivo only
Route of administration	I.V infusion	Oral
Onset	Immediate	1-3 days
Duration	3-5 h	2-5 days
Mechanism	Anti-thrombin-anti-activated factor Xa	Interfere with hepatic synthesis of factors II, VII, IX &X
antidote	Protamine sulfate	Vitamin K and fresh frozen plasma
Lab control	aPTT	Prothrombin time-INR
Crossing placental	No (safe in pregnancy)	Yes (unsafe)

V. Discussion

Signs of warfarin potassium overdose and toxicity

- Any unusual bleeding
- Blood in stool or urine
- Excessive menstrual bleeding
- Bruising
- Excessive nose bleeding
- Excessive gum bleeding
- Persistent oozing from superficial injuries
- Bleeding from the tumor, ulcer, or any other lesions
- Infrequent reactions as skin necrosis, purple toe syndrome, alopecia, urticaria, dermatitis, fever, anorexia, nausea, diarrhea, abdominal cramps, congenital disabilities, and abortion.

Warfarin potassium can be used alone and is appropriate to treat myocardial infarction, but it is associated with the risk of bleeding. The importance of thrombosis in the pathogenesis of heart diseases is well established; the process includes both coagulation system and platelet conditions. Patients with myocardial infarction who survive have a risk of death by 15% to 20% or having a rebound within 2 to 5 years, a proof that substantiates the rationale of secondary antithrombotic prophylaxis.

The anticoagulant warfarin potassium has two enantiomers metabolized by liver microsomal enzymes CYP450; R-warfarin potassium is metabolized primarily by CYP1A2 to 6-hydroxy warfarin potassium and 8-hydroxy warfarin potassium. R-warfarin potassium isomer is also metabolized by CYP3A4 to 10-hydroxy warfarin. S-warfarin potassium is metabolized mainly by CYP2C9 to 7-hydroxy warfarin potassium. The efficiency of warfarin potassium is affected particularly when the metabolism of the S-warfarin potassium enantiomer is altered.

Warfarin has been studied extensively in many controlled clinical trials. Drug interactions occur due to plasma protein displacement, interactions with the CYP450 enzyme system, or drug metabolism interference. The importance of liver microsomal enzymes genes such as CYP2C9 and VKORC1 genes to the patient-specific dose of warfarin has been established. Warfarin even interacts with natural products like ginseng, as there are documented interactions between alternative therapies and warfarin. Some people who take warfarin use medicinal plants OTC as they consider them a safe medication and do not interact with other prescribed drugs. This issue is very vital; especially with drugs that have a narrow therapeutic index, such as warfarin. Herbal products which may increase the risk of bleeding or even

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potentiate the risk of bleeding resulting from warfarin therapy are angelica root, anise, arnica flowers, capsicum, chamomile, clove, fenugreek, garlic, ginger, ginkgo Biloba, licorice root, onion, parsley, quassia, and turmeric.

Interpretation of the available information on warfarin-herb interactions is complicated because nearly most of these data are based on in vitro studies, studies on animals, or individual case reports. More studies are required to confirm the clinical significant of these interactions.

There is strong evidence that alternative therapy, primarily herbal medicines, has interactions with warfarin. Pharmacists and other health care professionals should ask their patients before prescribing warfarin about any current intake of herbal medicines.

Many drugs and foods interact with warfarin, including antibiotics, central nervous system drugs, and cardiac drugs. The danger is that substances can increase the warfarin effect as an anticoagulant. But on the other hand, gamma carboxylase polymorphisms and factor VII genes do not participate in predictive models of warfarin dose.

The single contribution of both CYP2C9 and VKORC1 polymorphism account for respective about 27% and 22% of maintenance dose variability (17). Therefore, the aggregate variability of warfarin potassium dose is explained by these two genes approaching 50% provided that other genetic factors.

Synthetic preservatives, benzalkonium chloride, are potent inhibitors of warfarin potassium for the CYP2C9 gene, producing unpredictable effects of warfarin potassium therapy (18). The impact of the treatment is measured by many variables, including drug interactions, illnesses, patient's history, dietary or GIT features that interfere with vitamin K efficacy and bioavailability, and physiological variables which affect the synthetic or metabolic fate of the vitamin K-dependent coagulation factors. So, genetic factors must be considered as they can be ideal while all these variables are stable.

Finally, CYP2C9 genotyping may not be helpful in some races like African-Americans or even as a marker for the long-term anticoagulation once the optimum and stable dose is reached (19).

VI. Conclusions

Indeed, it is needed to have a significant number of samples are to conduct clinical trials of this compound to determine its efficacy and activity on a large scale. But according to its chemical and biological properties, it is possible to expect its nature, mechanism of action, and its safety rather than warfarin sodium. The use of warfarin potassium has a well-known bleeding risk, although it can be used to treat patients with heart diseases or any cardiovascular disorders like thrombosis.

Generally, the role of any anticoagulant in secondary prophylaxis against myocardial infarction, for instance, is well established. Although using warfarin sodium or potassium has superiority rather than other oral anticoagulants like aspirin, aspirin is used widely.

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The Genomics of Liposarcoma: A Review and Commentary

By Hannah Beird, Alexander J. Lazar & Danh Truong
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Liposarcomas are Adipocytic Soft Tissue Sarcomas- Soft tissue sarcomas (STS) are malignancies that show mesenchymal and neuroectodermal differentiation and thus most often resemble supportive and connective tissue including fat, blood vessels, muscle, bone, tendons, and nerves. Over 70 subtypes of sarcomas exist and pathologists have classified these broadly according to the degree to which they resemble differentiated cell types (Figure 1)[1]. This review will focus on the most common subset of STS in adults, "liposarcoma", which are tumors with histological features of specialized fat cells. Liposarcoma are broken down into several subtypes. The four with the highest incidence are: well-differentiated liposarcoma (WDLPS), dedifferentiated liposarcoma (DDLPS), myxoid liposarcoma (MLPS), and pleomorphic liposarcoma (PLPS)[1]. Overall survival is highest for MLPS, followed by WDLPS and DDLPS, and then PLPS[2-4] (Figure 2). While WDLPS occurs predominantly in the deep soft tissues of the limbs and retroperitoneum, DDLPS is located mostly in the retroperitoneum. MLPS and PLPS are preferentially located within the limbs[5]. Despite these broad categories, liposarcoma can also have mixed phenotypes and is often further subdivided into even more rare entities with other ultra-rare features. For instance, pleomorphic MLPS has attributes of both PLPS and MLPS[6,7].

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The Genomics of Liposarcoma: A Review and Commentary

Hannah Beird [α], Alexander J. Lazar [σ] & Danh Truong [ρ]

I. Liposarcomas are Adipocytic Soft Tissue Sarcomas

Soft tissue sarcomas (STS) are malignancies that show mesenchymal and neuroectodermal differentiation and thus most often resemble supportive and connective tissue including fat, blood vessels, muscle, bone, tendons, and nerves. Over 70 subtypes of sarcomas exist and pathologists have classified these broadly according to the degree to which they resemble differentiated cell types (Figure 1)[1]. This review will focus on the most common subset of STS in adults, "liposarcoma", which are tumors with histological features of specialized fat cells. Liposarcoma are broken down into several subtypes. The four with the highest incidence are: well-differentiated liposarcoma (WDLPS), dedifferentiated liposarcoma (DDLPS), myxoid liposarcoma (MLPS), and pleomorphic liposarcoma (PLPS)[1]. Overall survival is highest for MLPS, followed by WDLPS and DDLPS, and then PLPS[2-4] (Figure 2). While WDLPS occurs predominantly in the deep soft tissues of the limbs and retroperitoneum, DDLPS is located mostly in the retroperitoneum. MLPS and PLPS are preferentially located within the limbs[5]. Despite these broad categories, liposarcoma can also have mixed phenotypes and is often further subdivided into even more rare entities with other ultra-rare features. For instance, pleomorphic MLPS has attributes of both PLPS and MLPS [6,7].

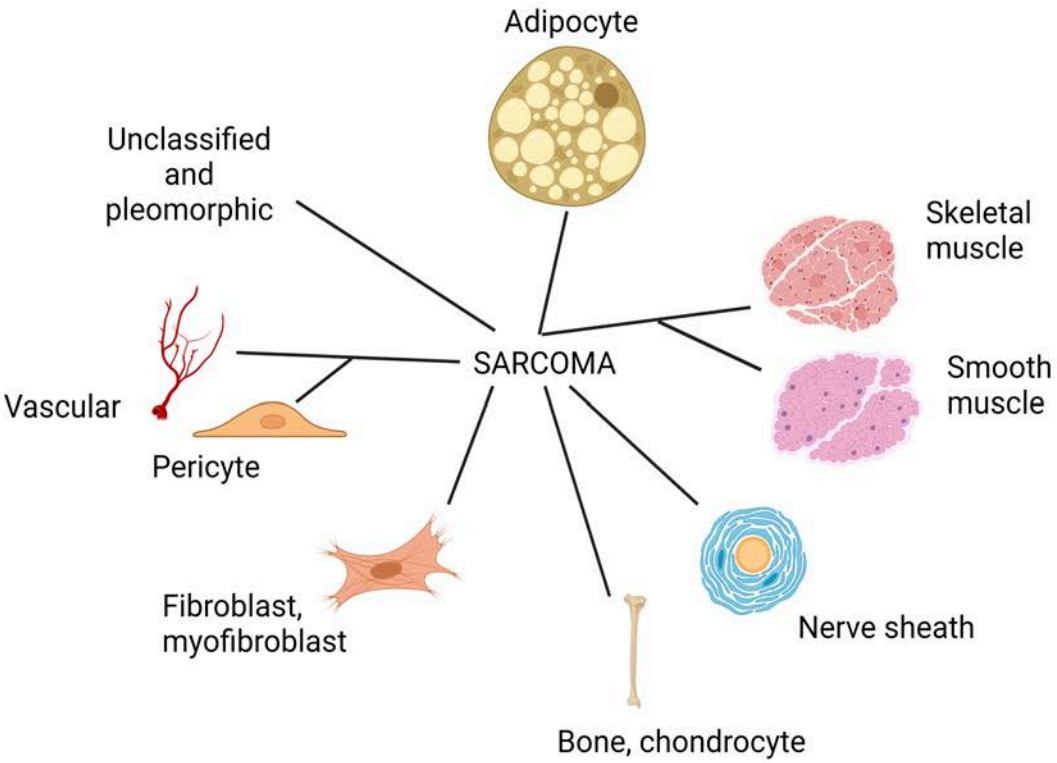

Figure 1: The Taxonomy of Soft Tissue Sarcoma. Sarcomas are classified according to pathologically defined tissue differentiation states. Liposarcomas are the adipocytic tumors

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II. Liposarcoma Genomic Classifications

STS have lower average somatic point mutation burdens than epithelial cancers[8]. When examining their karyotypic characteristics, they are classically divided into two major groups: complex and simple[9,10]. The liposarcoma subtypes WDLPS, DDLPS, and PLPS belong to the group of complex karyotypes, which are cells that have undergone steady and constant accumulation of multiple genomic copy number alterations, chromosomal anomalies and various types of rearrangements over time. This genomic instability is ongoing and occurs as a result of aberrations in genes involved in DNA repair, DNA replication and cell cycle regulation such as *TP53*[11]. The complex karyotypes in these liposarcomas are likely to have arisen from mutations in the *TP53* pathway. Both WDLPS and DDLPS have near universal amplification of chr12q, a region that includes *MDM2*, which is a gene that directs the protein degradation of *TP53*. For PLPS, recurrent mutations in *TP53 (7%)* and losses of *RB1* occur[12,13].

Behavior and changes in the microenvironment can create a permissive context under which liposarcoma form. For instance, over expression of the immune-related cytokine IL-22 in a mouse on a high fat diet led to the only reported spontaneous formation of WDLPS in a mouse model[14]. This implies that the relationship between the microenvironment and tumor may already be established when liposarcoma first form. This would explain why patient-derived WDLPS models have been difficult to establish as this dependence is still not well understood. Since chromosomal imbalances restrict the environment in which cancers can grow[15], the genomic instability that follows could then solidify this dependence.

Those sarcomas with simple karyotypes are nearly diploid; their driver events are typically fusion transcripts expressed via reciprocal chromosomal translocations. In the clinic, these diagnostic fusions are detected by fluorescent in situ hybridization (FISH), fusion panels, and reverse transcription polymerase chain reaction (RT-PCR). MLPS is an example of a liposarcoma with a simple karyotype and that is fusion-driven. It is mostly diploid and defined by a recurrent translocation between chromosomes 12 and 16: t(12;16) (q13;p11) that results in a fusion protein *FUS-DDIT3*.

III. Degree of Adipocytic Differentiation are Pathologic Markers of Liposarcoma

Each liposarcoma subtype resembles different stages of adipocytic differentiation (Figure 2). This was first illustrated in an unsupervised analysis of gene expression patterns found in WDLPS, DDLPS, MLPS, PLPS, benign lipoma and normal fat[16]. Three clusters formed: the first included normal fat, lipoma, and WDLPS; the second contained DDLPS and PLPS; and the third included only MLPS. In a complementary study, gene expression profiles of these four major liposarcoma subtypes were compared with those of human mesenchymal stem cells that were undergoing differentiation into mature fat. Each liposarcoma subtype resembled different stages in this process that were akin to their degree of differentiation[17]. For instance, DDLPS expressed genes that were comparable to those that at day 7, which reflects stem cells in their early stages of differentiation, only starting their commitment to becoming fat as compared to cells at day 21, when maturation is almost complete. In support of this, 16 genes from the PPARγ signaling pathway that leads to adipocytic terminal differentiation were significantly lower in DDLPS than in normal fat[18]. On the contrary, WDLPS was more similar to cells at day 21, when differentiation is almost complete. PLPS closely resemble cells at day 10 while MLPS or round liposarcoma resembled those at day 14. These expression patterns imply that the degree of dedifferentiation of liposarcoma can be related to survival, with higher degree of differentiation leading to improved survival.

DNA methylation patterns also reflect these differences in differentiation states. When examining DNA methylation states in 80 various sarcomas in an unsupervised manner, each liposarcoma subtype formed a distinct group[19]. Several distinguishing genes are related to adipocytic differentiation. One example is *NNAT*, which induces the activation of adipocytic transcription factors CREB and CEBP family[20]and was significantly methylated (hypermethylation) and upregulated in MLPS than in normal fat and other sarcomas[19,21]. Decreased methylation (hypomethylation) and downregulation of *NNAT* was observed in DDLPS and PLPS, which likely results in a more dedifferentiated state. Another example is the *CDKN2A* gene, whose CpG island methylation levels are shared by PLPS, DDLPS, and non-neoplastic fat, but not MLPS[19]. In addition, *ALDH1A3* is involved in the oxidative degradation of lipids and may contribute to cancer stem potential. A strong negative correlation between the methylation of *ALDH1A3* and its expression levels was found across several sarcoma subtypes, with the strongest hypermethylation and down regulation for MLPS[19].

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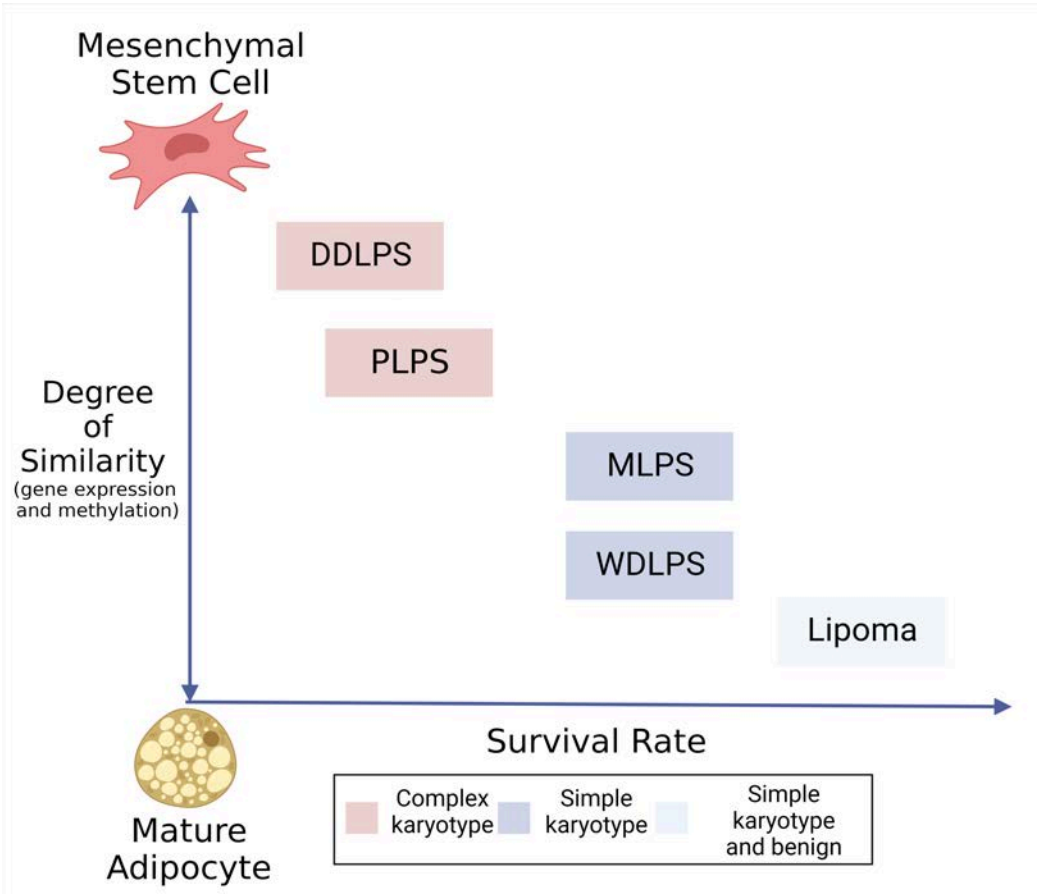

Figure 2: Survival and differentiation states of various liposarcoma subtypes

Dedifferentiated liposarcoma has the worst outcome, followed by pleomorphic, round-cell, then well-differentiated, and finally myxoid liposarcoma[22]. Gene expression and DNA methylation patterns in lipoma and liposarcoma subtypes are similar to those seen during the various stages along the differentiation pathway of mesenchymal stem cells as they progress towards becoming mature adipocytes. In concordance with these observations in liposarcoma, a recent hallmark of cancer – phenotypic plasticity – was recently added describing mechanisms associated with disrupted differentiation. The mechanisms are divided into three classes – dedifferentiation of mature cells to a progenitor or stem-like state, blocked differentiation preventing progenitor cells from maturing, and trans differentiation enabling switching between lineages. It is likely that dedifferentiation and blocked differentiation occur in the various liposarcoma subtypes and that these two mechanisms are intertwined and held in place through mutations or epigenetic patterns.

IV. Liposarcoma Formation through Genetic Loss

Patients with Li-Fraumeni syndrome and retinoblastoma have germline mutations of *TP53* and *RB1*, respectively, which leads to the formation of various tumor types, including high incidences of sarcomas as second and concurrent malignancies [23]. In Li-Fraumeni patients, these include liposarcoma, which occur less frequently than other sarcoma subtypes such as osteosarcoma, leiomyosarcoma (LMS), and rhabdomyosarcoma [24,25]. In like manner, retinoblastoma patients also occasionally develop liposarcoma, the majority of sarcoma risk being bone tumors, fibrosarcoma, rhabdomyosarcoma, and pleomorphic sarcomas[23]. Therefore, there is evidence that these canonic cancer genes are responsible for driving liposarcoma initiation.

Sarcoma tumor initiation by mutation of *TP53* and *RB1* tumor suppressor genes have been demonstrated *in vivo*. Genetically engineered mouse or rat models of *TP53* mutants develop sarcomas, namely angiosarcoma, osteosarcoma, and rhabdomyosarcoma with high levels of genomic instability[26-30]. Deletion of both *TP53* and *RB1* genes in mice leads to lower tumor generation time, resulting in greatly reduced survival than is seen when each gene is mutated alone[31]. When both *TP53* and *PTEN* are simultaneously deleted specifically within adipose tissue, spontaneous generation of all four subtypes of liposarcoma occur[32]. This model underscores the importance of the *TP53* and *PI3K/AKT* pathways in the initiation of liposarcoma, which may be partly due to the way in which they activate the Notch signaling pathway[33]. The effect of the

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PI3K/AKT pathway on tumor initiation would also explain why *PIK3CA* amplification by fluorescent in situ hybridization (FISH) is associated with older age, larger tumor size, and shorter disease-free survival duration in liposarcoma, without distinction for a particular subtype[34]. These models also illustrate how compounding gene losses can affect the nature and aggressiveness of the liposarcoma that is formed. This is further supported by the higher number of gene losses in DDLPS as compared to WDLPS[35].

TP53 and *RB1* also alter the ability of mesenchymal cells to differentiate. Knocking out *TP53* in mesenchymal stem cells (MSC) prevents the expression of PPARγ, a key gene in directing adipocytic differentiation[36]. Instead, these MSC cells become more prone to osteogenic differentiation[36]. Without *RB1*, stem cells can no longer differentiate efficiently[37]. RB1 either pushes osteogenic differentiation in MSCs through RUNX2 or prevents adipocytic differentiation by inhibiting PPARγ[36,38].

Since Li-Fraumeni is an example of a syndrome with germline predisposition to developing multiple types of cancer including liposarcoma, there may be other germline risk factors to be identified. Out of 4,432 unique liposarcoma records in SEER (1973-2015 cohort), 2968 (0.00063%) had a recording of other concurrent cancers. Liposarcoma have concurrent diagnoses in ovarian cancer[39], hereditary nonpolyposis colorectal cancer[40], Muire-Torre syndrome[41], multiple myeloma[42] and CLL (also our recent unpublished data and infiltration in TCGA-SARC sample)[8]. Identifying these predisposition genes will enable us to interpret mutations in sporadic cases, as illustrated by the discovery of the telomere protection gene, *POT1*, as predisposing to angiosarcoma and cardiac sarcomas[39].

V. Telomeres in Liposarcoma Survival and Persistence

Strategies to sustain cell survival include the elongation of chromosome ends: the telomeres. There are various Telomere Maintenance Mechanisms (TMM) including reactivation of the telomerase enzyme that serves to lengthen telomeres or the process of Alternative Lengthening of Telomeres (ALT) that employs homologous recombination methods to lengthen short telomeres. Activating mutations within the *TERT* promoter that encodes telomerase occur in a subset of MLPS[43]. On the other hand, inactivating mutations and copy number losses in genes involved in ALT (*ATRX DAXX*) are detected in a subset of all liposarcomas, most frequently in DDLPS[44]. Several assays are used to assess the activity of ALT within cells, which include: pulse field gel electrophoresis, terminal restriction fragment (TRF) Southern-blot analysis to measure telomere lengths, quantification of single-stranded circular DNA structures (C-circles) consisting of telomeric CCCTAA repeats, and immnofluorescence to identify the presence of ALT-associated promyelocytic leukemia bodies (APB). In all subtypes of liposarcoma, patients with ALT positivity as measured by these assays have worse progression-free and disease-specific survival rates[44-47]. DDLPS is often the subtype cited with more ALT+ than WDLPS[48].

VI. Well-Differentiated (WDLPS) and Dedifferentiated Liposarcoma (DDLPS)

Precursor or immature adipocytes are termed, "lipoblasts"[49]. Their gene expression patterns are most similar to those of nonmalignant adipocytes[50]. Well-differentiated (WDLPS) and dedifferentiated (DDLPS) liposarcomas are distinguishable from benign lipoblastoma and lipoma through karyotyping and breakpoint mapping. Lipoblastoma can have histological similarities with liposarcoma but is discriminated by an inversion involving the *PLAG1* gene on chr8[51,52]. Both lipoma and liposarcoma can have rearrangements or alterations on chr12. However, the breakpoints in lipoma appear to be more distal than in MLPS, WDLPS, and DDLPS[53] with rearrangements involving *HMGA2*, rather than amplification of the entire gene. Therefore, the breakpoint location serves to identify disease type and severity within the adipose tissue. More recently, lipomas were shown to have low mutation burden, low copy number alterations (CNA) and share mutations with liposarcoma in *APC*, *RYR2*, and *MAPK7*[54].

There is evidence that WDLPS and DDLPS share a common origin based on shared point mutations from which each subtype develops in an evolutionary divergence[35,55]. There are patients who transition from WDLPS to DDLPS and very rarely, others who go from a diagnosis of DDLPS to WDLPS. In fact, each liposarcoma tumor is a mixture of both subtypes with one dominating over the other at different times. This common origin and plasticity are attributed to the presence of extraneous supernumerary ring or rod chromosomes within the nucleus, called "neochromosomes", amidst otherwise diploid-looking genomes. The neochromosomes are also common in atypical lipomatous tumors and have occasionally been reported in lipoblastoma[56-58]. Whole genome sequencing of two isolated neochromosomes from a liposarcoma cell line revealed that they have no true centromeres and are therefore unstable[59]. Upon closer molecular assessment using copy number microarrays and spectral karyotyping, these neochromosomes are derivations of chr12q13-15 along with other chromosomes, most commonly chr1q21-22 and chr6q23[60-62]. The observation of chr12 amplifications in both WDLPS and DDLPS is nearly universal[63]. A minimum number of 20 copies per cell was observed

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using fluorescent in situ hybridization on the region that includes *MDM2* and neighboring gene *CPM*[64].

Out of the four current theories on the formation of these neochromosomes[65], two have evidence that they are likely the primary source of genetic heterogeneity within liposarcoma tumors (Figure 5). The first is that chromosome shattering events, called, "chromothripsis", generated these neochromosomes. This suggests that this transformative event may have selected for cells with chr12 as their primary backbone, which promoted cell survival[59]. This selection would seem most likely due to the most amplified genes: *MDM2* and *CDK4*. MDM2 inhibits the tumor suppressor TP53, thereby circumventing the cell's rescue signals during DNA damage to repair without proceeding through the cell cycle (G1 and G2 arrest) and any signals towards apoptosis that would cause the aberrant cell to die. CDK4 would allow for unimpeded and enhanced progression through the cell cycle. The manner in which these chromosome pieces are stitched together into a neochromosome appear random. Therefore, just as no two snowflakes are alike, it is conceivable that the number and content of neochromosomes in each liposarcoma cell would not be the same and would change with each cell division in the same way that mitochondrial DNA populations are altered in each daughter cell. The second theory is based on whole genome data of two DDLPS specimens that did not exhibit any features of chromothripsis[1]. In this study, the authors postulate that the neochromosomes are the result of progressive rearrangements and amplification. Both models are mutually exclusive and may delineate particular subsets of WDLPS and DDLPS. Following the generation of neochromosomes, either linear or circular breakage-fusion-bridge amplification (BFB) would lead to the multiple copies of the neochromosomes that are common to WDLPS and DDLPS[66]. Since BFB events do not always cause the exact same breaks within a chromosome, the daughter cell of any given neochromosome-containing parental cell is likely to be different (Figure 5). This was demonstrated using a CRISPR-based ecTag method in glioblastoma spheroids[67]. Amplification of oncogenes in extra chromosomal DNA may be the shortest route to heterogeneity than amplification of these genes within intact, autosomal chromosomes[67]. Hence, there is vast heterogeneity within the population of liposarcoma cells, supporting the early observations that both WDLPS and DDLPS contain all four CD34/CD36 adipose markers by flow cytometry, with each of the four populations present at different proportions[68]. The high level of heterogeneity is likely the reason treatment strategies are difficult to design. In addition, the triggers of transition or predominance of one subtype over the other is still unclear. Multiomic RNA and ATAC sequencing with spatial deconvolution may aid in tracking the mutation and environmental triggers as shown in recent studies in breast cancer and glioma[69].

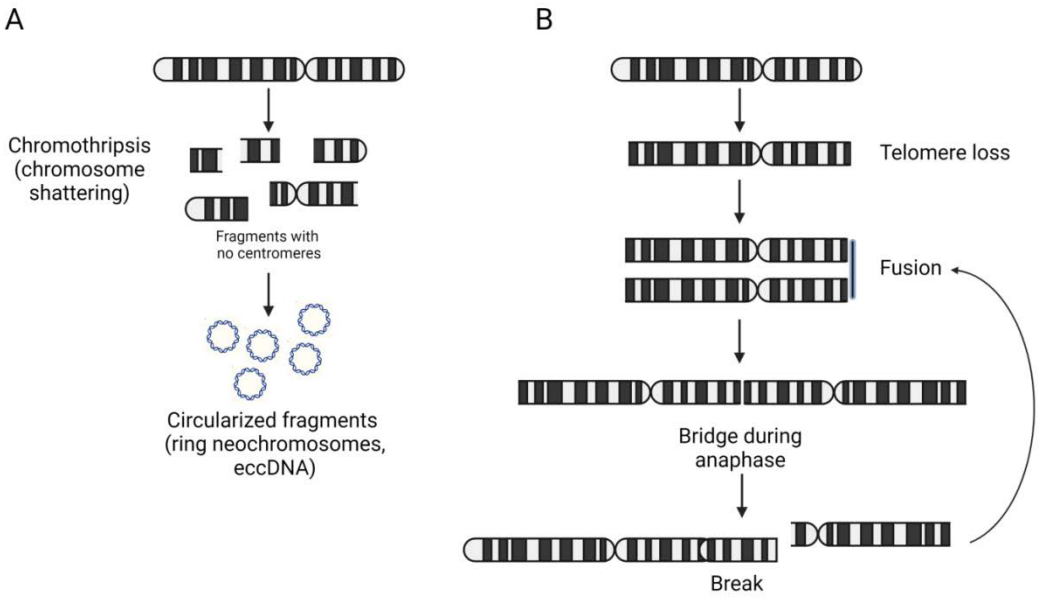

Figure 3: Neochromosome formation in liposarcoma. A. Chromothripsis leads to chromosome fragments that then circularize into neochromosomes. B. The Break-Fusion-Bridge pathway that generate rod neochromosomes. These rods have the potential to circularize into ring neochromosomes

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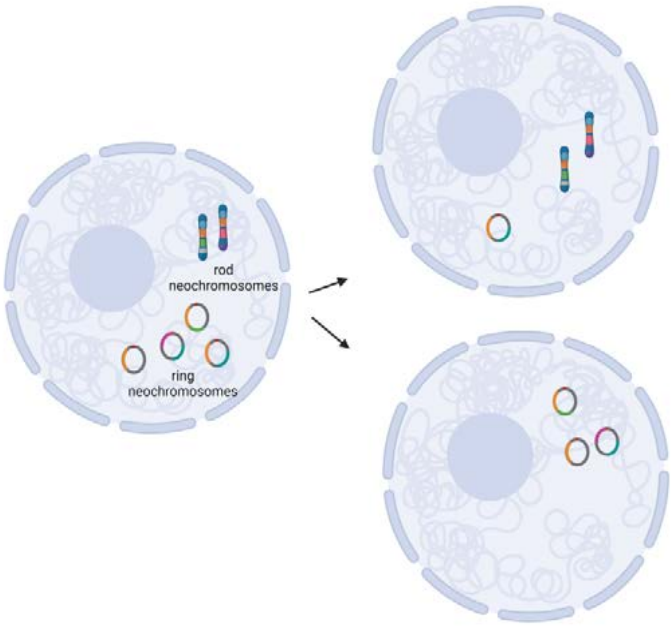

Figure 4: An illustration of the heterogeneity within an individual WDLPS or DDLPS due to the presence of neochromsomes. These nuclear neochromosomes are made up of different fragments from various chromosomes, chr12 being the most common and selected for (orange). The lack of true centromeres in these neochromosomes leads to the high probability of unequal segregation during mitosis, much like the random inheritance of mitochondrial DNA in daughter cells

Various genes within the region of chr12q amplification (*MDM2*, *HMGA2*, *YEATS4*, *FRS2*, *CPM*, *DDIT3*, *PTPRQ*) are implicated in the adipocytic differentiation pathways and in cancer progression. The degree to which each of these genes contribute to liposarcoma formation and progression is yet unclear. Evidence supporting roles for these genes is summarized below and in Tyler et al.[70].

MDM2: The N-terminal region promotes adipocyte differentiation through activation of CREB transcription at the expense of myogenesis in *P53*$^{-/-}$*; mdm2*$^{-/-}$ mouse embryonic fibroblasts[71]. *Mdm2* adipocyte-specific knock-in (*Mdm2*-AKI) mice have increased white adipose tissue dysfunction, weight gain and insulin resistance when fed a high-fat diet[72].

CPM: CPM was significantly increased genes in early stages of differentiation when inducing adipogenesis in bone marrow derived human mesenchymal stem cells[73], adipose tissue-derived human mesenchymal stem cells[73], and adipose-derived stromal cells[74]. Amplification that included *CPM* was observed in a large majority of WDLPS and DDLPS patient samples (78%, 39/50)[13]. CPM distinguishes WDLPS and DDLPS from lipoma through having higher protein levels than benign lipoma and normal fat tissue[13]. Knockdown using small interference RNA (siRNA) reduced cell proliferation, cell growth, colony formation, migration and invasion while increasing apoptosis in two of the DDLPS cell lines tested[13]. This finding was recapitulated in eight liposarcoma cell lines that had undergone a genome-wide CRISPER knockout screen (DepMap 22Q2 release)[75,76]. There, *CPM* was second most enriched dependency for viability among all the liposarcoma lines.

DDIT3: DDIT3 (CHOP/GADD153) is a chromatin remodeler that is expressed highly during the last stages of adipocytic differentiation from lipoblasts to adipocytes[77]. When over expressed in primitive sarcoma cells (fibrosarcoma) cells, DDIT3 can induce liposarcoma phenotypes[78]. It is expressed at the protein level in WDLPS, DDLPS, MLPS, PLS, and lipoma[79]. It blocks adipocytic differentiation by direct dominant negative inhibition of CEBP proteins from their target sites as well as preventing the accumulation of CEBPA in cells[80].

FRS2: FRS2 serves to recruit FGF, thereby facilitating FGFR signaling[81]. FGFR signaling is also active during differentiation of mesenchymal stromal cells[82] and human pre-adipocytes[83,84]. However, FRS2 inhibits adipocytic signaling in bone marrow stromal cells in 3D culture[85].These differing responses to FGFR signaling in cells according to environment and cell type that is receiving the signal may explain why not all liposarcoma have amplification of this gene.

HMGA2: FGF signaling also plays a role in *HMGA2* expression. *HMGA2* is a transcription factor that has relatively low expression in adult tissues as compared to embryonic and mesenchymal stem cells[86,87]. Thus, it is

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important for proper development of multiple tissues and has high expression in the first three hours of adipogenesis of 3T3-L1 preadipocytes before decreasing in subsequent stages[88]. FGF signaling by adipocytic stem cells can induce HMGA2 expression[89]. Once turned on, it can bind Rb1 to displace HDAC1 from Rb/E2F at their binding sites, leading to activated E2F1 and cell cycle progression[90]. It is upregulated in lipomas and transgenic mice that overexpress *HMGA2* result in hyperplasia of white adipose tissue[91,92]. These data suggest that HMGA2 alone cannot induce tumor progression and may only provide the proliferative context under which liposarcoma form.

PTPRQ: PTPRQ is a protein phosphatidylinositol phosphatase (PIPase) whose over expression would prevent adipocyte differentiation from mesenchymal stem cells[93]. Gain in PTPRQ on chr12q21 was observed in 46% of DDLPS[8].

YEATS4: By inhibiting the promoters of p14 and p21, YEATS4 (GAS4) represses the p53 pathway[94]. Knockdown of YEATS4 in non-small cell lung cancer cells leads to increased expression of p21, p53 and PARP cleavage[95].

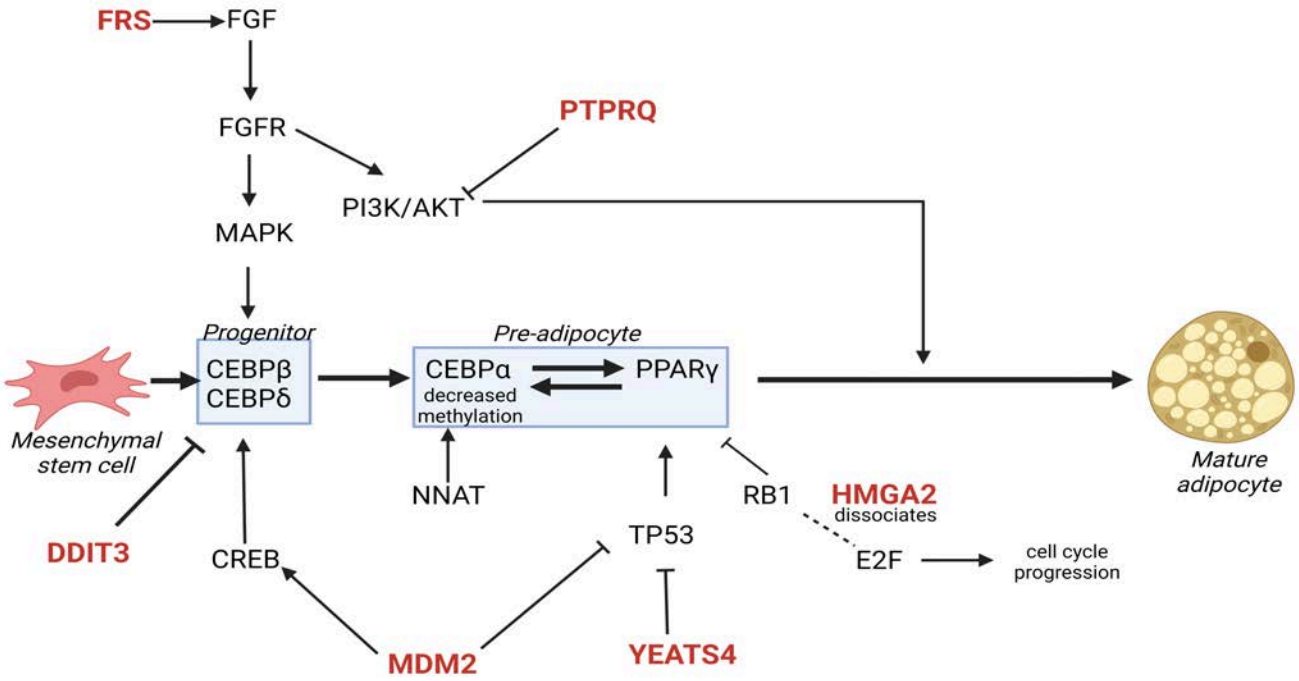

Figure 5: The role of genes from chr12 (in red) that are frequently amplified in WDLPS and DDLPS in adipocytic differentiation and tumor growth. The differentiation state of the tumor cell may affect the impact of these genes. Therefore, the selection of these genes for amplification may be determined by the cell differentiation state. *FRS* and *DDIT3* have documented activities affecting the *CEBP* transcription factors that direct earlier adipocytic progenitors while *YEATS4*, *HMGA2*, and *PTPRQ* appear to affect the later pre-adipocytic stages

Besides chr12q, other copy number alterations involved in adipocyte differentiation are aberrant in WDLPS and DDLPS. Loss of methylation within the promoter of *CEBPA* (chr19q13) may explain the lower expression of *CEBPA* in DDLPS than in WDLPS[66]. Gains in chr17p11 in DDLPS result in additional histologic features that are akin to UPS[96]. Gains in oncogenes that block adipocytic differentiation have been seen: *JUN* (chr1p32)[97] and *YAP1* (chr11q22)[98,99]. Lipid metabolism may be aberrant in DDLPS as losses and subsequent lower expression of genes such as *PLIN2* (chr9p22), *LIPE* (chr19q13), *DLAT* (chr11q23-24), and *ACAD8* (chr11q23-24) occur more often in DDLPS as compared to other sarcoma types[8,96]. Rearrangement of *SYT1* (calcium channel) was observed in WDLPS[100].

The level of heterogeneity and genomic complexity delineates differences between WDLPS and DDLPS. DDLPS have a higher number of point mutations that appear to be caused by the genome editing protein APOBEC (mutation signatures COSMIC2 and 13)[8]. However, these point mutations may not contribute to the etiology of disease (passenger events) as the number was positively associated with age, largely nonfunctional, and not known to be cancer drivers[8,35]. DDLPS also harbor higher number of rearrangements and copy number alterations than WLPS[35,101]. In fact, the frequency of somatic copy number alterations was highest in DDLPS when compared against LMS, undifferentiated pleomorphic sarcoma (UPS), synovial sarcoma (SS), and malignant

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peripheral nerve sheath tumor (MPNST)[8]. DDLPS has overall poorer survival, likely due to these increased burdens of mutations and copy number alterations[2-4]. Further reduced local relapse-free survival was observed in DDLPS patients with loss of chr19q13 or chr9p22-24 or chr17q21[96]. When integrating both copy number and methylation alterations in a set of DDLPS (TCGA-SARC), disease-specific survival rate was significantly longer in one subgroup, cluster K3 (chr6q25.1 amplified and fewer unbalanced chromosome segments than K2) that shared a particular pattern of copy number alterations. Clusters K1 (JUN amplified) and K2 (TERT amplified and chromosomally unstable) had worse survival than K3[8]. This group had the lowest levels of immature dendritic cell infiltration. Overall, the study suggested that copy number alterations and methylation impacted survival and may be used as predictive biomarkers for DDLPS.

VII. Myxoid Liposarcoma (MLPS)

Myxoid liposarcoma are the most common liposarcoma in young patients under age 22[6]. The characteristic pathological features of myxoid liposarcoma are stellate spindle cells, signet-ring lipoblasts, "crow's feet" vascular network[102], and markers of immature adipogenicity[103]. Transitional areas of increased cellularity can occur[104] with other patterns: round cell, pseudoacinar, lipoblast-rich, island, lipomatous, stromal hemangiopericytoma-like characteristics[105]. The presence of small blue round cells in more than 5% of the tumor is considered the "round-cell" subtype[104], which is more aggressive with poorer prognosis that metastasizes more frequently to the bone rather than to other tissue sites[104].

Within a background of a mostly quiet karyotype, the diagnostic molecular feature found in more than 95% of tumors is the reciprocal translocation t(12;16) (q13;p11): FUS-DDIT3 (TLS-CHOP)[106]. There are at least 10 known variants, of which the major categories involve breakpoints nearexon 5 or exon 7/8 of *FUS*, while other breakpoints occur after exon 4, 8, 13 in *DDIT3*[107]. These breakpoints eliminate the RNA-binding domain of FUS, which is then replaced by the DNA-binding domain of DDIT3 along with the rest of DDIT3 that includes a leucine zipper dimerization domain[108]. Only variants with breakpoints near or after exon 13 have an intact RNA-binding domain from *FUS* in the fusion protein.

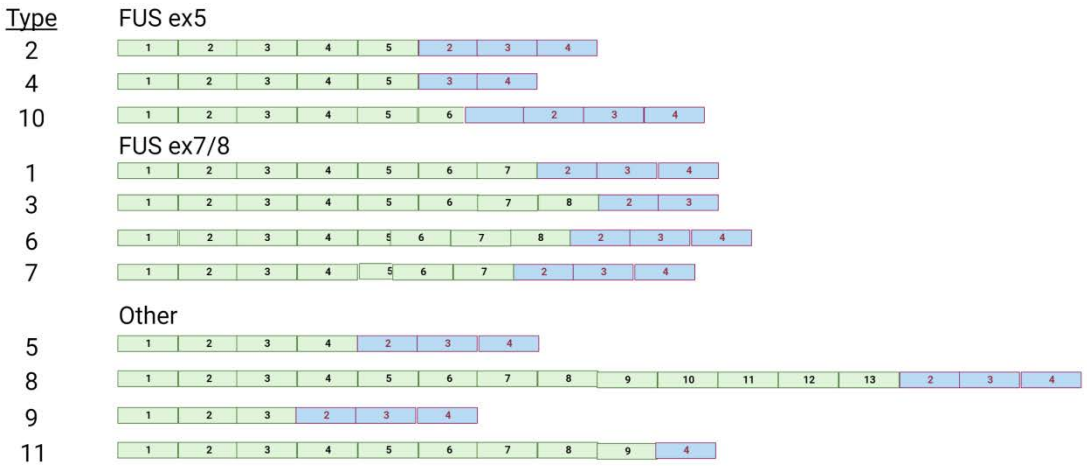

Figure 6: FUS-DDIT3 fusion transcript isoforms. FUS exons are shown in green, while DDIT3 exons are in blue

The FUS-DDIT3 fusion can transform mesenchymal cells in mice[109], partly by stimulating eIF4E expression, which results in down regulation of the PPARγ and C/EBPα pathways, thereby inhibiting adipocytic differentiation[110]. It can also activate the IGFR1/PI3K/AKT pathway[110] and repress miR-486, which may result in upregulation of PAI-1, a molecular that is involved in tumor invasion and metastasis[111]. An alternative mouse model demonstrated that expressing FUS-DDIT3 under a mesoderm promoter *Prx1* within a p53 null background results in synergy in tumor formation[112]. This may explain the poorer likelihood of survival in myxoid liposarcoma patients with *TP53* mutations[113]. Therefore, mutation in *TP53* may contribute to a more aggressive tumor in the context of this translocation.

An alternative translocation event, EWSR1-DDIT3 (t(12;22)(q13;q12)), occurs in a minority of patients (4-5% in both pediatric and adult) (4-5%) with at least 4 known transcript isoforms[114]. *FUS* and *EWSR1* are functionally interchangeable since either gene fused to DDIT3 induced tumors in a xenograft model[115]. In fact, *FUS* and *EWSR1* are paralogs, belonging to the FET family of general RNA-binding proteins that also includes TAF15[116]. Together, these proteins appear to interact in a single complex[117,118] with a myriad of roles in RNA splicing, association with RNA helicases, DNA damage response, miRNA processing, RNA transport,

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and possibly others[116]. Arginine methylation by the PRMT family, namely PRMT1, regulates their nucleo-cytoplasmic localization and binding to DNA[119].

Rearrangements involving FUS and EWSR1 with other C-terminal partnersoccur in various other cancer and sarcoma subtypes. For instance, FUS-ATF1 was found in an angiomatoid fibrous histiocytoma[120,121], FUS-ERG occurs in acute myeloid leukemia[122,123], FUS-BBF2H7 in low grade fibromyxoid sarcoma[124], and FUS/EWSR1-KLF17 in myoepithelial tumors[125] In an analogous way, EWSR1- WT1 and EWSR1-FLI1 occur in Ewing Sarcoma and desmoplastic small round cell tumor (DSRCT), respectively[126,127]. Since the FET family forms the N-terminal partner, the C-terminal part of the fusion may affect protein interactions, differentiation state and the cell type that ultimately becomes malignant[116]. For myxoid liposarcoma, DDIT3 may affect fat differentiation, while other partners such as WT1 for Ewing Sarcoma and DSRCT (EWSR1-WT1 translocation) influence other tissue types. In addition, point mutations of *FUS* are frequently observed in patients with amyotrophic lateral sclerosis (ALS)[128-130]. These mutations disrupt the nuclear localization sequence so that FUS remains in the cytoplasm[131]. Therefore, the type of mutation within a specific gene can drive different, unrelated diseases. Also, the mechanisms by which similar translocations diseases depends on the partners involved. Thus, comparisons of these mechanisms in various disease types may elucidate the roles of each translocation partner in disease generation as well as inform as to whether we can combine patients with these different diseases into basket trials for novel therapeutic options.

Other distinguishing genomic features have been described for myxoid liposarcoma. The presence of the testis antigen NY-ESO-1 is thought to differentiate myxoid liposarcoma from other myxoid tumors[132]. *TERT* promoter mutations are the most frequent in myxoid liposarcoma as compared to other sarcomas[43]. Activating mutations in *PIK3CA* are the most common in myxoid liposarcoma as compared to other major liposarcoma histotypes[34], with greater incidence in round cell myxoid liposarcoma[133]. These mutations appear to be mutually exclusive with *PTEN* loss and IGF1R expression[133]. In addition, patients with *PIK3CA* mutations in the helical or kinase domains have a shorter disease-specific survival than those with wild type *PIK3CA*[134]. Lower survival is also associated with methylation of the p14(ARF) promoter that leads to lower expression of ARF[135,136]. Higher proliferative activity in MLPS is associated with high levels of β-catenin[137] whereas growth through angiogenesis may be positively influenced by the hypermethylation and down regulation of the extracellular matrix glycoprotein EFEMP1, as compared to normal fat[19].

VIII. Pleomorphic Liposarcoma (PLPS)

The definition of this particular subtype is the presence of pleomorphic lipoblasts[138]. It is found frequently in the extremities of older adults, with those within upper extremities having poorer survival[139]. This subtype excludes the distinguishing mutations found in the other subtypes described above: no fusions involving DDIT3 and no consistent amplification of MDM2[140]. It has a more complex karyotype than other liposarcoma subtypes[141,142], which may explain why these patients have the shortest survival of all liposarcoma subtypes. This complex karyotype nature of PLPS may form the basis for its pathologic and copy number profile resemblance to undifferentiated pleomorphic sarcoma (UPS)[138,143]. Both had gains in: 1p, 1q, 5p, 19q, and 20q and recurrent losses in 1q, 2q, 3p, 4q, 10q, 11q, and 13q (including *RB1*). When comparing PLPS karyotypes among multiple complex karyotype pleomorphic sarcomas[144], the frequency of chromosomal aberrations was the fewest in pleomorphic liposarcoma. Thus, these may not be as advanced in complexity and severity as other pleomorphic sarcoma. Missense *TP53* mutations within exons 5-9 were found in 60% of the 31 cases that were examined[141,142], low levels of Rb1, and other features such as phyllodes of the breast[145] which occur more frequently in women with Li Fraumeni Syndrome (*TP53* germline mutation).

IX. Current and Future Genomics

a) *Single Cell Sequencing*

Despite the recent advances in understanding liposarcoma biology, there is still much to unravel in order to find effective targeted therapies for recurrent or metastatic lesions. Questions remain on how we can effectively explore themes within the complex heterogeneity of liposarcoma including degrees of adipocytic differentiation, mixed phenotype or clonal subtype, and cell of origin, which may enable avenues to potential therapeutics. Recently, single-cell sequencing (SCS) has made a dramatic impact on the field of cancer by revealing novel cell/differentiation states, exploring inter- and intra-tumor heterogeneity, and discovering rare cell populations previously undetected. Since Macosko et al. and Klein et al. developed Drop-Seq and in Drop respectively in 2015, approximately 14,534 articles were found using the keywords 'single-cell' and 'sequencing' to search in PubMed[146,147]. Among those articles, 68 contained the word 'sarcoma', and 5 contained 'liposarcoma'. This suggests that SCS is not being effectively used to explore sarcoma and liposarcoma genomics given the prevalence of SCS within the last decade. In the following section, we will discuss applying various SCS technologies to liposarcoma genomics, describe the common pitfalls when approaching liposarcomas, and

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examine the intersection of SCS and liposarcoma clinical care. Where liposarcoma-specific data are limited, we will extrapolate lessons learned from the cancer field and other sarcomas.

The democratization and commercialization of SCS have led to stable platforms for cancer research. The most widely used modality – transcriptomics or single-cell RNA-sequencing (scRNA-seq) –can profile gene expression for thousands of cells within a single experiment. The gene expression profile for each cell can be used to characterize and catalogue the cellular taxonomy of the tumor as well as define novel states or subtypes in cancer cells. Importantly, scRNA-seq has been used to detect rare subpopulations of cells including cancer stem cells and circulating tumor cells. For epigenomics, the most popular method is single-cell ATAC (assay for transposase-accessible chromatin) sequencing (scATAC-seq), which is used to measure the chromatin accessibility in single cells. Lastly, for genomics, single-cell DNA-sequencing (scDNA-seq) can be used for copy number alteration profiling, mutations, and clonal evolution. Additional layers of information can be studied through single-cell multiomics, where subsequent technologies can be used on cells of the same specimen followed by computational integration methods to combine the data or within the same cell where cellular barcodes link different -omics data.

While somatic hallmarks can be detected with techniques such as WES, SCS enables deeper exploration of mutations in the subpopulations within the tumor. Since both WDLPS and DDLPS contain amplifications within chr12q regions, SCS could be used to detect copy number alterations (CNAs) to separate malignant cells apart from normal cells and determine the clonal substructure of the malignant cells. This could enable understanding the cell of origin and how degrees of adipocytic could affect tumor burden. Technologies like Tapestri (Mission Bio) and Single-Cell CNV (10x Genomics) can directly detect CNAs by scDNA-seq. Recent work by the Navin group demonstrated that CNAs between scDNA-seq from single cells when merged together and bulk whole-exome-sequencing (WES) had high concordance for patients with triple negative breast cancer (TNBC). Pearson correlation showed a mean of 0.871 across five different matched patient data sets[148]. A key limitation with this approach is that possible mutations for TNBC patients must be well-known in advance. This data feeds into a custom targeted panel of all known mutation sites for scDNA-seq, which greatly reduces the cost when compared to an unbiased panel. Advantages to using this approach, aside from the cost reduction, is enabling high-throughput single-cell analysis of clonal diversity within patients and understanding of possible clonal substructures. Key questions this could answer for WDLPS and DDLPS would be to understand the clonal evolution or transition, if it occurs, between WDLPS and DDLPS.

As an alternative to scDNA-seq, there are multiple software packages that can infer CNAs from scRNA-seq data. This has the advantage of utilizing the more popular scRNA-seq with the addition of evaluating gene expression[149,150]. The currently available software packages used to infer CNAs from scRNA-seq data are: InferCNV, CaSpER, and CopyKAT[149,151,152]. They operate under the assumption that CNAs are correlated with increasing or decreasing gene expression and that by fitting a mixture model to the data set, confounding factors from normal gene expression fluctuation could be removed. Work on synovial sarcoma, an aggressive neoplasm driven by the SS18–SSX fusion, demonstrated that CNAs could be detected using infer CNV on the scRNA-seq data and that the inferred CNAs matched the data from WES[153]. The limitations with using software to infer CNAs from scRNA-seq is dependent on the model used with each software varying in detection of CNAs. In addition, normal reference cells may be required as an input and, in some cases, malignant and normal reference cells may not be easily distinguishable from the gene expression data alone. Nonetheless, these inference methods could determine tumor heterogeneity and enable identification of patient-specific features that are not found in the gene expression data.

Importantly for MLPS, which is driven by FUS-DDIT3, and in some cases, EWSR1-DDIT3 fusion, SCS can detect and quantitate fusions or structural rearrangements. However, this depends on the sequencing chemistry. There are four popular chemistries for generating sequencing reads – full-length, 3', 5', and tagmentation. 3' and 5' sequencing have been popularized by 10x Genomics, since these chemistries can easily enable profiling of up to 10,000 cells. However, these chemistries have high bias for 3' or 5' read coverage. This hinders the ability to detect mutations such as SNPs, indels, and rearrangements that may not exist at either 3' or 5' ends. In that regard, full-length mRNA profiling does enable in-depth sequencing capable of genotyping and detecting mutations. One such method that uses full-length mRNA sequencing is the SMART-seqwork flow (Takara Bio). However, SMART-seq has much lower throughput compared to 3' and 5' SCS. It requires fluorescent-activated cell sorting (FACS) to sort single-cells into wells of a 96-well plate. This does have an added benefit of cell typing the cells prior to sequencing if the cell type specific surface markers are well-expressed. Recently, SMART-seq was employed to detect the *SS18-SSX* fusion transcripts in synovial sarcoma[153]. A common problem in SCS is annotating malignant cells v. normal cells. In this case, the presence of the fusion transcript was used to delineate malignant cells from normal cells. As for MLPS, since there are at least 10 known variants

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of the translocation, as known through synovial sarcoma, SMART-seq could easily identify the variants, while having the added benefit of transcriptomic data for each cell linked to any one variant. Importantly, regardless of grade, MLPS has potential to metastasize. A key question to explore using SCS would be to identify if there exists a cell state or subclone within the lesion that has a higher propensity for metastasis.

Feasibility of using SCS with fatty tissues, such as liposarcoma, is an important issue to resolve. Two major concerns with adipocytes are their large and fragile nature, which has proven to be a problem with SCS technologies. An alternative strategy to certain SCS methods, like scRNA-seq, which typically uses whole cells, is to use the nucleus – termed single-nucleus RNA-seq (snRNA-seq)[154]. SnRNA-seq has previously been leveraged for various mouse and human adipose tissue[155-157]. Recently, an atlas of white adipose tissue demonstrated that only snRNA-seq was capable of sequencing and detecting adipocytes, which were not present in the scRNA-seq data from the same tissue[158]. Interestingly, while many of the other cells within the microenvironment were also present in snRNA-seq, there were also a lower abundance of endothelial and immune cells. Overall, this suggests that sequencing liposarcoma, where cases with WDLPS tend to be fattier, may require nucleus rather than whole cell. In that regard, techniques using scDNA-seq or scATAC-seq should not be affected since the nucleus is typicallly the default input.

b) Cell-Free tumor DNA in Liposarcoma

Detection of possible recurrence or metastasis in patients with liposarcoma that have undergone complete resection can be difficult and costly. Because there are no diagnostic biomarkers associated with possible recurrence or metastasis, clinical examinations with frequent imaging throughout the body is the only alternative.

Recently, cell-free tumor DNA (ctDNA) has emerged as a novel method to interrogate cancer biology and etiology in a feasible manner by profiling tumor-derived materials, such as blood, cerebral spinal fluid, and urine[159]. CtDNA often contain genetic material that had been shed from tumor cells, where such materials should reflect the tumor genome in some capacity. At a molecular level, somatic mutations, copy number alterations, methylation, and point mutations can be detected in ctDNA by sequencing methods. In that regard, ctDNA is a useful diagnostic tool that could detect early diagnosis and predict tumor burden and activity and overcome the hurdles of traditional diagnostic methods such as imaging and traditional biopsies.

Given that MLPS has a well-defined molecular diagnostic feature, the FUS-DDIT3 or the alternative EWSR1-DDIT3 translocation, ctDNA has recently been used to monitor disease activity of patients with MLPS[160]. Quantification of ctDNA of the t(12;16) breakpoint for multiple patients using digital droplet PCR demonstrated a correlation of ctDNA concentration with tumor volume and stage. Upon resection there was an observed drop-off of t(12;16) ctDNA, while recurrence or metastases was associated with an increase of t(12;16) ctDNA concentration.

Interestingly, unlike MLPS where the translocation was detected by ctDNA, genotyping WDLPS and DDLPS was more difficult. While these tumors harbor amplifications resulting in multiple copies of *MDM2, CDK4,* and *HMGA2*, the method for detection by digital droplet PCR in a recent study was not nearly as sensitive[160]. CtDNA derived from the peripheral blood from five WDLPS/DDLPS patients were collected and primers for *MDM2* and a control gene, *EIF2C1*, were used to genotype. The *MDM2*/*EIF2C1* ratio was 1.21 (range of 1.14-1.38), whereas health patients had a ratio of 1.09 (range of 0.69–1.41), which had no statistical significance, suggesting that PCR may not have enough specificity and sensitivity to detect the CNAs. On the other hand, a separate study used shallow whole-genome sequencing, which is well-established for genotyping with low-coverage, to detect *MDM2* in ctDNA from the plasma of WDLPS and DDLPS patients[161]. Interestingly, only two out of three DDLPS patients had readily detectable *MDM2* amplification. This seemed to correlate with tumor size, where the undetected patient had a tumor size of 14 cm v. 19 and 25 cm. Moreover, no WDLPS patients had detectable *MDM2* amplification in ctDNA, perhaps due to the lower cellularity content of these tumors as opposed to DDLPS. In addition, a longitudinal study showed that *MDM2* levels decreased after tumor resection. Overall, these data suggests that *MDM2* amplification could be detected for DDLPS patients by shallow whole-genome sequencing from the plasma.

While PLPS is an aggressive sarcoma with high recurrences, it does not have a unifying genetic alteration that could be easily detected for disease monitoring. Over 50% of patients diagnosed with PLPS will eventually have metastatic disease[22]. A study evaluating PLPS for biomarkers failed to identify prognostic biomarker for patients whose follow-up information was available (n=22)[12]. Despite the lack of distinctive genetic alterations, patient-specific gene variants found within the ctDNA could be a possible avenue for detecting residual disease or possible recurrence. One strategy would be to perform deep NGS sequencing on tumor tissue from surgical resection to discover patient specific alterations. Paired analysis of patient plasma from ctDNA using a targeted approach, like molecular tag-based sequencing, may reveal concordant mutations with the tumor tissue that could be used for disease monitoring during follow-up. In a recent study that monitored patient-specific ctDNA

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across a diverse set of tumors, the authors found that for patients (n=40) with three or more longitudinal time points the patient-specific ctDNA had a correlation with tumor burden in 16/19 (85%) patients with partial response and overall in 27/40 (68%) patients[162]. On the other hand, use of cancer antigens only correlated with tumor burden in 19/40 (47.5%) patients, suggesting a lower utility than patient-specific ctDNA.

Outside of somatic mutations, detection of DNA methylation in ctDNA may offer an alternative modality for monitoring tumor burden and recurrence. Whole-genome bisulfite sequencing (WGBS) can detect DNA methylation throughout the genome. Importantly, methylation patterns greatly differ between malignant and normal cells, and could be used to distinguish between different cancer types. Certain sarcomas, such as synovial sarcomas, had unique methylation patterns that was relatively uniform[8]. On the other hand, DDLPS had 3-4 methylation patterns that overlapped with undifferentiated pleomorphic sarcoma and gynecologic leiomyosarcoma. Nonetheless, detecting methylation in ctDNA has utility for monitoring tumor burden. The Circulating Cell-free Genome Atlas (CCGA; NCT02889978)is a prospective, multi-center, observational study that uses machine learning to detect cancer type and tumor burden from ctDNA[163]. By WGBS, methylation signatures could robustly identify several cancer types with high specificity. Importantly, they found that WGBS of ctDNA outperformed WGS, which detected somatic mutations, and targeted mutation panels in classifying cancer types. Because methylation is more pervasive than mutations, it may enable lower limits of detection compared to detection limits for somatic mutations detected through WGS or targeted ctDNA panels[164]. A clear limitation in this study is the small number of sarcoma patients included. Another limitation is that not all participants were asymptomatic, could inform the utility of DNA methylation for disease monitoring. Studies including asymptomatic patients were still ongoing.

X. Summary

In summary, WDLPS, DDLPS and PLPS have complex genomics due to either formation or propagation of neochromosomes or complex rearrangements and copy number alterations. These mutations lead to high levels of heterogeneity generating mixed tumor phenotypes, which can be difficult to classify. The altered genes, which are selected for during tumor evolution, drive the perpetual survival and continued growth of immature or poorly differentiated dipocytes. Unlike the other liposarcoma subtypes, MLPS is characterized by a translocation, where the N-terminal partner, DDIT3, in a healthy context plays an important role in regulating adipogenic differentiation. However, in the setting of MLPS, the fusion protein may instead act as an aberrant transcription factor inhibiting adipogens is and maintaining immature adipocyte. In addition to genetic alterations, tumor development and formation may be influenced by exogenous factors including surrounding the tissue microenvironments and tissue inflammatory state as well as endogenous factors including *TP53*, *RB1*, and *PI3K/AKT/PTEN* pathways. A summary of the current therapies against these drivers and other genes are reviewed in Keung and Somaiah and Tyler et al. [70,165]. Overall, the severity of disease appears to be strongly influenced by higher degrees of genetic alterations and poorer differentiation. Insights into mechanisms of phenotypic plasticity – dedifferentiation or blocked differentiation – may enable better understanding on how to control differentiation in liposarcoma therapeutically. It is important to note that phenotypic plasticity is not a novel invention by cancer cells but rather a co-opt of latent mechanisms that are used by healthy cells to support tissue homeostasis[166].

The latest developments in tools and technologies, including SCS and ctDNA, will be fundamental in advancing biology, diagnostics, and molecular therapeutics. SCS may shed light on intertumoral heterogeneity and identify subclones with actionable gene targets. Utilizing ctDNA may enable a feasible method for diagnosis and disease monitoring where recurrence is a possibility. Most importantly, continued exploration of the genomics of liposarcoma should enable advances in drug development centered on the genetic alterations.

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Epigenetics Theoretical Limits of Synthetic Genomes: the cases of Artificials *Caulobacter* (*C. eth-2.0), Mycoplasma Mycoides (JCVI-Syn 1.0, JCVI-Syn 3.0 and JCVI_3A), E-coli and YEAST chr XII*

By Jean-claude Perez

Abstract- In (Venetz et al., 2019), authors rebuilt the essential genome of *Caulobacter crescentus* through the process of chemical synthesis rewriting and studied the genetic information content at the level of its essential genes. Then, they reduced the native *Caulobacter crescentus* native *Caulobacter* NA1000 genome sequence real genome (4042929 bp) to the 785,701-bp reduced synthetic genome. Here we demonstrate the existence of a palindromic-like mirror structure that exists in real genomes and disappears totally in the synthetic genome. This biomathematic meta-organization is based on characteristic proportions of Fibonacci numbers between DNA single strand nucleotides proportions TC/AG on the one hand and TG/AC on the other hand. In both cases, we suggest that this meta-structure enhances the three-dimensional cohesion of the two DNA strands of the genome. We then generalize this study to the different synthetic genomes and synthetic cells published by the Craig Venter Institute on *Mycoplasma Mycoides* JCVI-syn1.0 (in 2010), JCVI-syn3.0 (in 2016) and JCVI-syn3A (in 2019). Finally, in the discussion section, we extend this study to synthetic genomes of *E-Coli* and *Yeast chromosome XII.*

Keywords: synthetic genomes, epigenetics, transposons, biomathematics, fibonacci numbers.

GJSFR-G Classification: DDC Code: 547.2 LCC Code: QD262

EPIGENETICSTHEORETICALLIMITSOFSYNTHETICGENOMESTHECASESOFARTIFICIALSCAULOBACTERCETH2OMYCOPLASMAMYCOIDESJCVISYN1OJCVISYN3OANDJCVI3AECOLIANDYEASTCHRXII

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Epigenetics Theoretical Limits of Synthetic Genomes: The Cases of Artificials *Caulobacter (C. eth-2.0), Mycoplasma Mycoides (JCVI-Syn 1.0, JCVI-Syn 3.0 and JCVI_3A), E-coli and YEAST chr XII*

Solved by standard Gammas, unvarying Deltas, uniform Epsilons. Millions of identical twins. The principle of mass production at last applied to biology
in « BRAVE NEW WORLD » Chapter 1, Aldous Huxley 1931
https://www.huxley.net/bnw/one.html

Jean-claude Perez

Abstract- In (Venetz et al., 2019), authors rebuilt the essential genome of *Caulobacter crescentus* through the process of chemical synthesis rewriting and studied the genetic information content at the level of its essential genes. Then, they reduced the native *Caulobacter crescentus* native *Caulobacter* NA1000 genome sequence real genome (4042929 bp) to the 785,701-bp reduced synthetic genome. Here we demonstrate the existence of a palindromic-like mirror structure that exists in real genomes and disappears totally in the synthetic genome. This biomathematic meta-organization is based on characteristic proportions of Fibonacci numbers between DNA single strand nucleotides proportions TC/AG on the one hand and TG/AC on the other hand. In both cases, we suggest that this meta-structure enhances the three-dimensional cohesion of the two DNA strands of the genome. We then generalize this study to the different synthetic genomes and synthetic cells published by the Craig Venter Institute on *Mycoplasma Mycoides* JCVI-syn1.0 (in 2010), JCVI-syn3.0 (in 2016) and JCVI-syn3A (in 2019). Finally, in the discussion section, we extend this study to synthetic genomes of *E-Coli* and *Yeast chromosome XII.*

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I. Introduction

The story which led to the development of the first synthetic genome JCVI-syn1.0 has its origins as far back as 1995, when Venter and his team published the sequence of *Mycoplasma genitalium* (Fraser, 1995) and (Sleator, 2010). In 2010, a 1079-kb genome based on the genome of *Mycoplasma mycoides* (JCV-syn1.0) was chemically synthesized and supported cell growth when transplanted into cytoplasm. (Gibson, 2010). In 2016, Hutchinson et al design, build, and test cycle to reduce this *Mycoplasma mycoides* genome to 531 kb (473 genes). JCV-syn3.0 retains genes involved in key processes such as transcription and translation, but also contains 149 genes of unknown function. Since 2012 the Synthetic Yeast Genome Project (Sc2.0 http://syntheticyeast.org/sc2-0/) results from a worldwide partnership, « Sc2.0 International Consortium team », members spanning 4 continents to provide remote mentorship and solve challenges associated with synthetic individual chromosome design features and assembly (Jee Loon Foo 2018). Read the analysis in §Discussion. In January 2019, Breuer et al. published a synthetic cell resulting from the synthetic genome JCVI-syn3A, a robust minimal cell with a 543 kbp genome and 493 genes, providing a versatile platform to study the basics of life. Simultaneously, in 2019, Venetz et al. reduced the native Caulobacter crescentus NA1000 genome sequence real genome (4042929 bp) to the 785,701-bp reduced synthetic genome Caulobacter ethensis-2.0 (C. eth-2.0). Finally, also in 2019 (Fredens, 2019), researchers published a synthetic genome of E COLI changing systematically genetic code equivalent codons. They replaced every occurrence of the serine codon TCG with AGC, every TCA (also serine) with AGT, and every TAG (stop) with TAA. Read the analysis in § Discussion.

In a completely different field, 30 years ago, we had just published the first 2 French books on Artificial Intelligence (AI) neural networks (Perez, 1988; Perez, 1989; Perez, 1990a). It is the exploration of our network FRACTAL CHAOS (Perez 1990c), (Pellionisz et al, 2012), (Perez§Montagnier, 2021), which will reveal a hyper-sensitivity of this network to successive ratios of

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Fibonacci numbers, for example 34/21 (Perez, 1990b). While the big project of sequencing of the human genome "HUGO" just begins, we have the intuition to look for ratios of Fibonacci numbers between the contiguous proportions of TCAG nucleotides of genes and small genomes available at that time (like HIV, mtDNA, viruses, bacteria, or small genes). We published a first article in 1991 (Perez, 1991; Marcer, 1992) demonstrating the evidence of such biomathematic structures (Perez, 1991). This discovery was completely published 22 years ago in the book "DNA decrypted" (Perez, 1997). This method, which the Nobel prize winner Luc Montagnier called "DNA supracode" (Fleaux, 1995), was used to search exaustively in DNA searched exhaustively in DNA sequences for remarkable proportions of Fibonacci numbers (https://en.wikipedia.org/wiki/Fibonacci_number) between nucleotides called "resonances": for example if a contiguous sequence of 377 bases TCAG is subdivided into 233 (C + A) and 144 (T + G), there is a resonance of CA / TG of length 377 (where 144, 233 and 377 are three Fibonacci numbers). In (Perez, 2017a), it is precisely such resonances CA / TG that characterize this optimality of the mtDNA genome of humans. It is still such resonances that are affected during mutations associated with cancers. In particular, we have analyzed this type of resonance in the 3 respective mtDNA genomes of humans, mice, and the famous naked mole rat as well as in more than a dozen other mammalian species.

In a comprehensive analysis of all (ALL) listed mutations of the human mitochondrial mtDNA genome associated with cancers : effectively, multiple mutations associated with the mitochondrial genome of tumor cells have been reported. An open question is whether these mutations are only the CONSEQUENCE of the cancer process or if, on the contrary, they would be a possible ORIGINAL CAUSE of the cancer genesis process. In a paper in preparation (Perez, 2019) we'll propose a generic and universal law (of a numerical nature) allowing us to detect and classify these mutations at the early stage of the genesis of the tumors. Finally, in (Perez 2019) we will present a generic law of prediction and classification of tumors by the simple analysis of the DNA SUPRACODE of the mitochondrial genomes associated with these tumors. In this upcoming article, we analyse all known somatic mutations listed all cancers combined. We then discover a global strategy of mutation of all these basic somatic mutations materialized by a numerical score which systematically increases in ALL the cases of elementary somatic mutations related to 91 referenced cases involved in 9 different cancers (prostate, pancreatic, colon, thyroid, bladder, breast, head § neck, meduloblastoma, ovarian) with a success rate of 100%. This predictive method should make it possible to categorize and classify the potential pathogenicity of tumors from the early stage. Particularly, we find an interesting symmetric property of resonances with very short periods: for example, the resonances 3 (1 TC 2AG) and 3 (2TC 1AG) correspond to the symmetrical beginnings of the Fibonacci and Lucas sequences. Similarly, the resonances 5 (2 TC, 3AG) and (3TC 2AG) correspond to the symmetrical beginnings of the Fibonacci and FibLuc[1] [2] sequences. By looking for these resonances in all the known tumor mutations of human mtDNA genomes applied to the genomes inherited by evolution of the RSRS mother sequence (EVE), it appears the functional role of such local resonances whose repercussion on the global scale of the genome becomes a indicator of early diagnosis of tumors.

It is this type of symmetry that we will generalize in this article by extending it to longer Fibonacci, and Lucas sequences.

Example 34 TCAG ==> 13 TC, 21 AG in one hand (regular)

and 34 TCAG ==> 21 TC, 13 AG in other hand (reverse).

II. Experimental Section

Part I: Genomes analysed

We will analyze 8 bacterial genomes, 3 real reference genomes, one transgenic genome, and four synthetic genomes.

==> The 2 Caulobacter genomes:

Name: NA1000 real

Reference: Caulobacter crescentus NA1000, complete genome

Publication: Venetz, 2019

Length: 4042929 bp
Access: native *Caulobacter* NA1000 genome sequence [National Center for Biotechnology Information (NCBI) accession no. NC_011916.1]
https://www.ncbi.nlm.nih.gov/nuccore/NC_011916.1

Name: Ethensis CETH 2.0

Reference: Synthetic Caulobacter sp. 'ethensis' strain CETH2.0 chromosome, complete genome

Publication; Venetz, 2019

Length: 785701 bp
Access:
https://www.ncbi.nlm.nih.gov/nuccore/CP035535

==> The 6 Mycoplasma Mycoides genomes:

[1] This sequence 3 2 5 7 12 19 31 50... results adding Fibonacci (1 2 3 5 8...) and Lucas (1 3 4 7 11...) sequences like : 2 (1+1), 5 (2+3), 7 (3+4), 12 (5+7)... Curiously, we discovered this sequence in (Perez, 2017b) resulting from stationary waves observed in DUF1220 repeat proteins in mammals brain coding DNA genomes.

[2] This sequence was also curiously used in new orleans jazz negro spiritual music (Parayon 2011).

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Name: MycRef
Reference: Mycoplasma mycoides subsp. mycoides strain izsam_mm5713, complete genome
Publication: Orsini, 2015
Length: 1192498 bp
Access:
https://www.ncbi.nlm.nih.gov/nuccore/CP010267.1?report=genbank
Name: JCVI-syn1.0
Reference: Synthetic Mycoplasma mycoides JCVI-syn1.0 clone sMmYCp235-1, complete sequence
Publication: Gibson, 2010
Length: 1078809 bp
Access:
https://www.ncbi.nlm.nih.gov/nuccore/296455217
Name: Capritrans
Reference: Mycoplasma mycoides subsp. capri str. GM12 transgenic clone tetM-lacZ, complete genome
Publication: Direct Submission
JOURNAL Submitted (14-MAY-2009) The J. Craig Venter Institute, 9702 Medical
Center Drive, Rockville, MD 20850, USA
Length: 1089202 bp
Access:
https://www.ncbi.nlm.nih.gov/nuccore/CP001621.1
Name: Capri real
Reference: *Mycoplasma mycoides subsp. mycoides SC str. PG1*
Length: 1211703 bp
Access:
https://www.ncbi.nlm.nih.gov/nuccore/NC_005364.2
Name: JCVI-Syn3.0
Reference: Synthetic bacterium JCVI-Syn3.0, complete genome
Publication: Hutchinson, 2016
Length: 531490 bp
Access:
https://www.ncbi.nlm.nih.gov/nuccore/CP014940.1
Name: JCVI-Syn3A
Reference: Synthetic bacterium JCVI-Syn3A chromosome, complete genome
Publication: Breuer, 2019
Length: 543379 bp
Access:
https://www.ncbi.nlm.nih.gov/nuccore/CP016816.2

Part II: Computing DNA Supra Code Resonances:
Let us consider the 2 digital sequences :
Fibonacci: 1 1 2 3 5 8 13 21 34 55 89
Lucas: 2 1 3 4 7 11 18 29 47 76

For any contiguous sequence of nucleotides, one will search for "resonance" or exact proportions of the TG / CA types then mainly TC / AG.

For example, if 34 TCAG bases are subdivided exactly into 13 TC bases and 21 AG bases, we will consider having discovered a TC / AG resonance of length 34. We will do the same for the search for Lucas resonances. The whole genome is explored by taking each of the positions as successive exploration points. On the other hand, the genome being circular, the analysis from the last pivots at the end of the sequence is looped back to the positions of the start nucleotides.

We will thus search for 2 symmetrical types of resonances:

Main resonances (or forward): Exp. 34 TCAG ==> 13 TC, 21 AG.

Inverse Resonances (or backward): Exp. 34 TCAG ==> 21 TC, 13 AG.

For each length of Fibonacci (or Lucas) 3 5 8 13 21 34 55 89, we memorize the respective accumulations of the forward resonances on the one hand, and backward on the other hand.

It appears then that these 2 values are very close in the case of REAL genomes, whereas they are very different in the case of SYNTHETIC genomes.

We will therefore consider very significant: The forward / backward ratios. Forward-backward spreads. Since the lengths of real and synthetic genomes are generally very different, we will weight the forward-backward differences by the respective lengths of the real or synthetic genomes.

III. Results

We analyse here, in one hand, Caulobacter crescentus NA1000 genome and synthetic genome Caulobacter ethensis-2.0 (C. eth-2.0), and, in other hand, Mycoplasma Mycoides JCVI-syn1.0, JCVI-syn3.0 and JCVI-syn3A.

Part I: Caulobacter crescentus NA1000 genome and synthetic genome Caulobacter ethensis-2.0 (C. eth-2.0).

The actual NA1000 genome being about 5 times longer than the synthetic genome C. eth-2.0, one might think that the comparison of these 2 genomes is skewed. However, in all the above results, we had already incorporated this difference by weighting the results by the length of the respective genomes.

TC/AG analysis:

Nota: All tables in this article are identical: each box contains 4 numerical values: 1/The number "L" of Fibonacci or Lucas constituting the length of the sub-sequence analyzed. 2/The cumulated volume of the corresponding resonances (n x L) in regular exploration (forward). 3/The cumulative volume of the corresponding resonances (L x n) in reverse (backward) exploration. 4/The ratio of the 2 values below regular/reverse.

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Table 1: TC/AG Fibonacci and Lucas analysis for real NA1000 genome and synthetic Caulobacter 2.0.

TC/AG Real genome NA1000				TC/AG Synthetic genome CAULOBACTER 2.0			
Fibonacci		Lucas		Fibonacci		Lucas	
3	1580254 1582201 0.9987694357	3	1582201 1580254 1.00123208	3	290047 293417 0.9885146396	3	293417 290047 1.011618807
5	1346353 1346512 0.9998819171	4	993497 994619 0.9988719299	5	239292 242875 0.9852475553	4	194318 198624 0.9783208474
8	924521 926003 0.9983995732	7	1186993 1186953 1.0000337	8	168546 172325 0.9780705063	7	209444 211890 0.9884562745
13	650861 652564 0.9973902943	11	661049 662784 0.9973822542	13	120565 124586 0.9677251055	11	123932 128203 0.966685647
21	377329 378580 0.9966955465	18	489878 492487 0.9947023982	21	74278 77737 0.9555038141	18	92499 96693 0.9566256089
34	188645 190539 0.9900597778	29	232050 233697 0.9929524127	34	40816 44246 0.9224788681	29	48895 52692 0.9279397252
55	71250 72070 0.9886221729	47	104152 104668 0.9950701265	55	18131 20974 0.8644512253	47	24743 27988 0.8840574532
89	19694 19535 1.008139237	76	30804 31161 0.9885433715	89	6300 8172 0.7709251101	76	9026 11226 0.8040263674

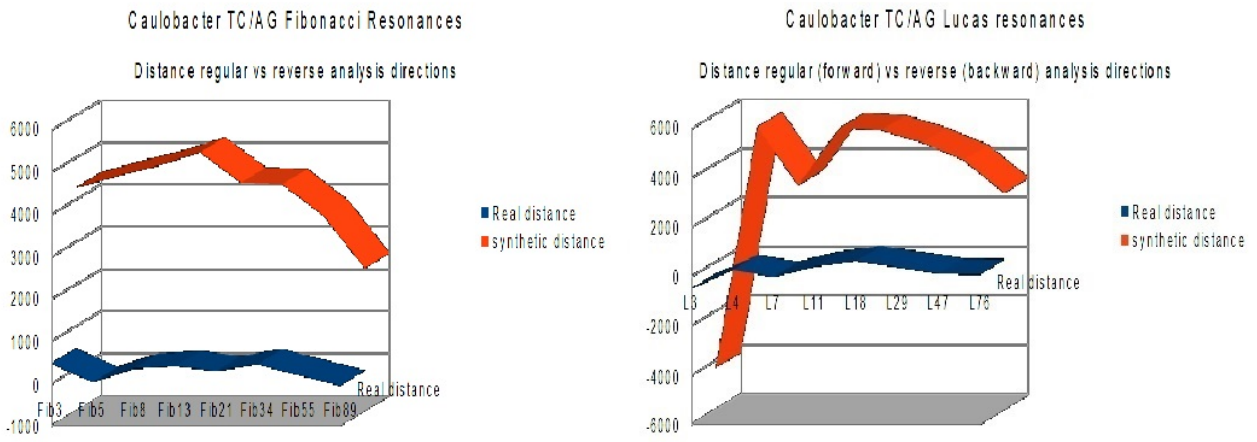

Figure 1: Comparing TC/AG Fibonacci and Lucas distances in real and synthetic Caulobacter genomes
(regular-reverse distances weighted by the length of the genome, see detailed data in suplementary materials)

example of weighting by the length of the genome computing here the case of synthetic caulobacterium genome (case of the first Fibonacci resonance of length = 3 nucleotides):

regular - reverse distance = 290047-293417 = ¯3370

weighting by the genome length : ¯3370÷785750 = ¯0.004288895959

normalization multiply by 1000000 = 1000000× ¯0.004288895959 = ¯4288.895959 = -4289

(see more details in suplementary materials page 2).

In the figure on the left, the average ratio of weighted distances by genome length between real genome and synthetic genome is 14.39 for TC/AG Fibonacci resonances (see supplementary materials).
For information, the same ratio related to Lucas TC/AG is = 14.345484

Computing details

real genome abs. Distances:	482	40	367	422	310	469	203	39
synthetic genome abs. Distances:	4289	4560	4810	5118	4403	4366	3619	2383

cumulating real genome abs. Distances: 2332

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cumulating synthetic genome abs. Distances: 33548
Ratio synthetic genome abs. Distances / real genome abs. Distances = 14.38593482

TG/AC analysis

Table 2: TG/AC Fibonacci and Lucas analysis for real NA1000 genome and synthetic Caulobacter 2.0.

TG/CA Real genome NA1000				TG/CA Synthetic genome CAULOBACTER 2.0			
Fibonacci		Lucas		Fibonacci		Lucas	
3	1607779 1614874 0.9956064684	3	1614874 1607779 1.00441292	3	296256 299993 0.9875430427	3	299993 296256 1.012614091
5	1349192 1358378 0.993237523	4	1011587 1022025 0.9897869426	5	244860 248873 0.9838753099	4	195156 199978 0.9758873476
8	915781 925976 0.9889899954	7	1174192 1180896 0.9943229548	8	169216 174299 0.9708374689	7	213326 216126 0.9870445944
13	642769 652548 0.9850141292	11	654997 665831 0.9837286038	13 120130 125243 959176		11	123819 128731 0.9618429127
21	384426 392873 0.9784994133	18	489376 497649 0.9833758332	21	73909 78870 0.9370990237	18	92670 96745 0.9578789602
34	209433 218299 0.9593859798	29	251102 259033 0.9693822795	34	42458 46179 0.9194222482	29	50145 53889 0.9305238546
55	91756 97158 0.9443998436	47	127067 133142 0.9543720239	55	20641 22427 0.9203638471	47	27305 29582 0.9230275167
89	31478 34303 0.917645687	76	45537 49636 0.9174188089	89	8188 9531 0.859091386	76	11334 12669 0.8946246744

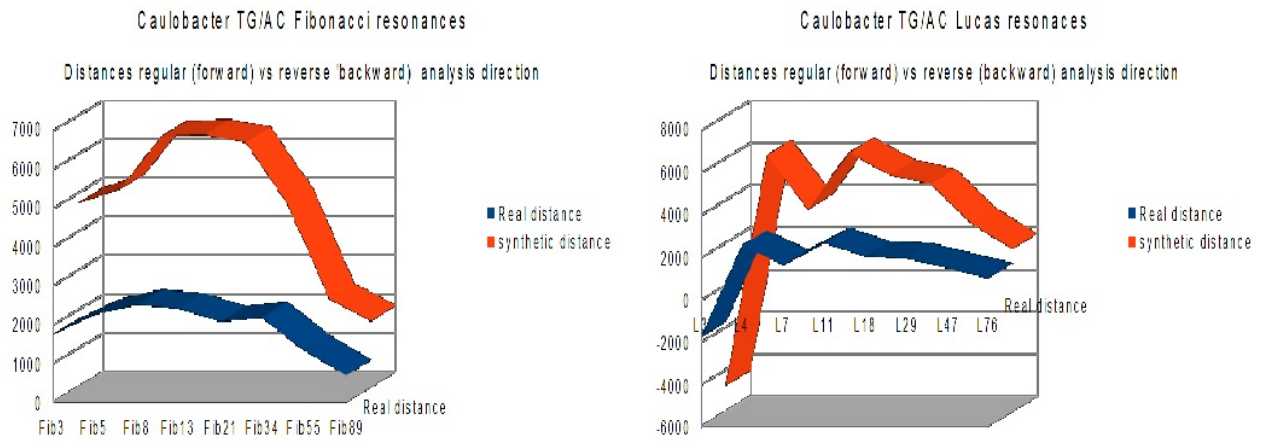

Figure 2: Comparing TG/AC Fibonacci and Lucas distances in real and synthetic Caulobacter genomes (regular-reverse distances weighted by the length of the genome, see detailed data in suplementary materials)

Part II: Mycoplasma Mycoides JCVI-syn1.0 (2010), JCVI-syn3.0 (2016) and JCVI-syn3A (2019)

In 2010, a 1079-kb genome based on the genome of *Mycoplasma mycoides* (JCV-syn1.0) was chemically synthesized and supported cell growth when transplanted into cytoplasm. (Gibson, 2010). In 2016, Hutchinson et al. design, build, and test cycle to reduce this *Mycoplasma mycoides* genome to 531 kb (473 genes). JCV-syn3.0 retains genes involved in key processes such as transcription and translation, but also contains 149 genes of unknown function. In the following section, we compare 6 (six) genomes : two reference real strain mycoplasma genomes including CAPRI strain, one transgenic building strain and the 3 strong JCV Labs ; synthetic genomes.

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Table 3: Comparing TC/AG Fibonacci analysis for 6 real or synthetic Mycoplasm genomes

Mycoplasm REF real genomes						Synthetic Mycoplasm genomes					
Natural reference genomes				Transgenic genome		Synthetic genomes					
Reference real strain		Reference real strain CAPRI		Transgenic CAPRI strain		JCVI-Syn1.0		JCVI-Syn3.0		JCVI-Syn3A	
3	425653	3	431863	3	389604	3	386328	3	191148	3	195794
	418009		424453		383147		380244		188330		192178
	1.01828		1.017457		1.0168525		1.016000		1.014963		1.0188158
6688		763		4		252		097		89	
5	349081	5	353999	5	322151	5	319643	5	158678	5	162644
	342772		348030		314644		312321		155189		158262
	1.01840		1.017150		1.0238587		1.023443		1.022482		1.0276882
5821		82		1		829		264		64	
8	249100	8	252787	8	228917	8	227112	8	112973	8	116120
	241395		244971		220387		218687		108400		110250
	1.03191		1.031905		1.0387046		1.038525		1.042186		1.0532426
864		817		42		381		347		3	
13	182285	13	184673	13	167319	13	166119	13	83100	13	85559
	173428		175894		158110		156734		77329		78424
	1.05107		1.049910		1.0582442		1.059878		1.074629		1.0909798
0185		742		6		52		182		02	
21	122345	21	123850	21	111834	21	110910	21	55296	21	57103
	114349		116312		103016		101748		50364		50837
	1.06992		1.064808		1.0855983		1.090045		1.097927		1.1232566
6278		446		54		996		091		83	
34	80074	34	81099	34	72890	34	72247	34	36064	34	37460
	73025		74515		66214		65356		31989		32353
	1.09652		1.088358		1.1008245		1.105437		1.127387		1.1578524
8586		049		99		909		539		4	
55	49907	55	50665	55	45287	55	44848	55	22000	55	22992
	44879		46176		40085		39272		19301		19368
	1.11203		1.097215		1.1297742		1.141984		1.139837		1.1871127
4582		003		3		111		314		63	
89	30152	89	30708	89	27153	89	26742	89	13030	89	13749
	26319		27108		22722		22243		10741		10743
	1.14563		1.132802		1.1950092		1.202265		1.213108		1.2798101
6232		125		42		881		649		09	

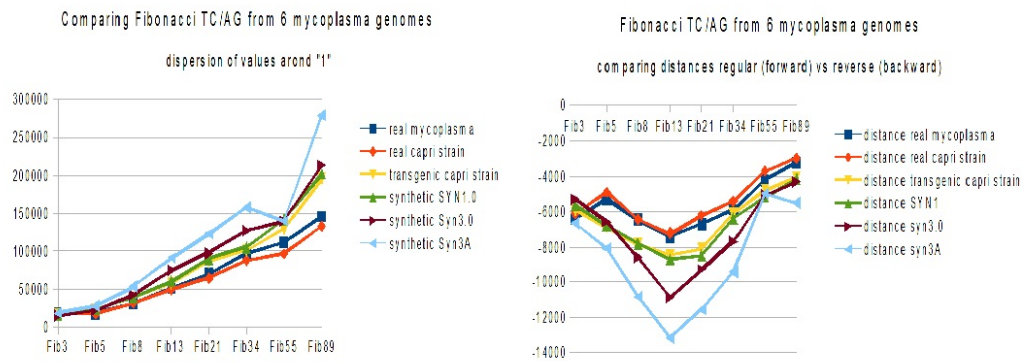

Figure 3: Left: Comparing TC/AG Fibonacci ratios from 6 mycoplasma genomes (relative values around 1), right: Comparing TC/AG Fibonacci distances from 6 mycoplasma genomes. (regular-reverse distances weighted by the length of the genome, see detailed data in suplementary materials)

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In summary of this double analysis it seems obvious that synthetic genomes disturb and destroy a characteristic dimension of real genomes. This property could concern the mathematical topology of the genome (Rapoport, 2018) and probably its fractal, dynamic, evolution, and three-dimensional structures.

IV. Discussion

a) Comparing real E COLI Genome and synthetic changing TAG by TAA stop codons

In (Fredens et al., 2019), researchers published a synthetic genome of E COLI changing systematically genetic code equivalent codons. They replaced every occurrence of the serine codon TCG with AGC, every TCA (also serine amino acid) with AGT, and every TAG (stop codon) with TAA, for a total of 18,214 replacements. Here we run a sample comparison of TG Fibonacci resonances changing stop codons TAG in TAA, then 7725 changes considering only TAG of the first codons reading frame ; In (Fredens et al., 2019), the sequences and genome design details used in this study are available in the Supplementary Data. Supplementary Data 1 provides the GenBank file of the *E. coli* MDS42 genome (NCBI accession number AP012306.1); Fredens's team systematically replaced every occurrence of the serine codon TCG with AGC, every TCA (also serine) with AGT, and every TAG (stop codon) with TAA, for a total of 18,214 replacements ; Not having access to the modified sequence of the synthetic genome yet, we simply changed all TAG codons to TAA codons, that is, 7,725 altered codons. We have limited this change to only the first reading frame codons.

Table 4: Comparing Fibonacci TG/AC from E-COLI real genome and E-Coli synthetic where all TAG codons are removed in TAA codons (1st codons reading frame only)

ECOLI reference wild type genome				ECOLI syn61 like where 7725 TAG ==> TAA			
3	1471002	1476399	0.9963444841	3	1458201	1484100	0.9825490196
5	1211718	1215554	0.9968442373	5	1204279	1221598	0.9858226683
8	852126	857586	0.9936332916	8	844110	865264	0.9755519703
13	612152	618151	0.9902952515	13	604631	625851	0.9660941662
21	385231	390203	0.9872579145	21	378106	397617	0.9509301665
34	222919	227478	0.9799585015	34	216935	233775	0.9279649235
55	107343	110152	0.9744988743	55	102252	114831	0.8904564099
89	43863	45199	0.9704418239	89	41531	47929	0.8665108807

Note, the TAG ==> TAA mutations (where G is mutated to A) does not affect the TC / AG structures, we have here to analyze the TG / AC structures.

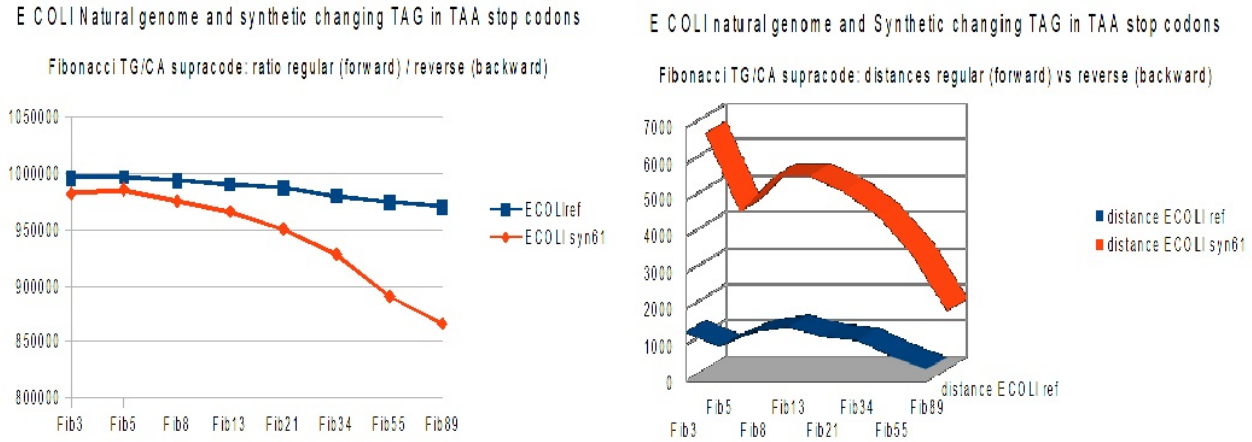

Figure 4: Left: Comparing TG/AC Fibonacci ratios in real and synthetic E-Coli genomes, Right: Comparing TG/AC Fibonacci distances in real and synthetic E-Coli genomes (regular-reverse distances weighted by the length of the genome, see detailed data in suplementary materials)

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b) Yeast Synthetic Genome, the case of the longer chromosome XII

Since 2012 the Synthetic Yeast Genome Project (Sc2.0 http://syntheticyeast.org/sc2-0/) results from a worlwide partnership, « Sc2.0 International Consortium team », members spanning 4 continents to provide remote mentorship and solve challenges associated with synthetic individual chromosomes design features and assembly (Jee Loon Foo, 2018).

Sources

synthetic yeast project

http://syntheticyeast.org/

7 chromosomes now synthetised

http://syntheticyeast.org/sc2-0-data/

Consorsium has successfully synthesized seven chromosomes. Check the following links to learn about details related to each finished chromosomes:

- synII synIII synV synVI synIXR synX synXII

In (Weiming Zhang et al., 2017) process building the whole synthetic chromosome XII.

Having not yet obtained the synthetic genome from the authors, we have limited here our study to the concatenation of all wild type PCRTags on the one hand and synthetic ones on the other hand. For example:

Forward wild type PCRTag : TGCTTGAACTGCAAATACAGGCCCACTC

Forward synthetic PCRTag : AGCTTGGACAGCGAAAACTGGACCTGAT

They published particularly all the wild type and synthetic PCR Tags.

The full PCR Tags are available online:

http://syntheticyeast.org/wp-content/uploads/2016/10/synXII_PCRtag.txt

Details: PCRTags

« PCRTags are alterations incorporated into most open reading frames (ORFs) (on average one per ORF, as some ORFs are too small and others contain multiple PCRTags). These are made by recoding a ~20bp segments of the coding region of an ORF to a different DNA sequence encoding the same amino acid sequence. PCR primer pairs can then be designed that will selectively amplify only the synthetic or wild type sequences. In this way, transformants that have incorporated a synthetic segment can be quickly scanned to ascertain that a complete substitution of the segment has occurred. PCRTags can also be used to monitor for the deletion of non-essential segments post-SCRaMbLE induction. » (from http://syntheticyeast.org/designs/alterations/pcrtags/).

We analysed 681 PCRTags of each 28 bp from wild YEAST XII and artificial SYN XII chromosomes. Then only resonances < 28 bp are to be considered in the following analysis.

We run 3 analysis :

Fibonacci sequence= 1 2 3 5 8 13 21 34 55 89

Lucas sequence= 1 3 4 7 11 18 29 47 76

FibLuc sequence= 5 7 12 19 31 50 81 131

Table 5: Comparing real and synthetic YEAST chromosome XII PCRTags with Fibonacci, Lucas and FibLuc resonances

YEAST XII real genome (681 wild type PCRTags)						Synthetic genome SYNXII (681 synthetic PCRTags)					
Fibonacci		Lucas		FibLuc		Fibonacci		Lucas		FibLuc	
3	6906	3	7073	5	5726	3	7133	3	7436	5	6195
	7073		6906		5649		7436		7133		5982
	0.976389		1.024181		1.0136307		0.959252		1.042478		1.0356068
0853		871		31		2862		62		2	
5	5649	4	4760	7	3094	5	5982	4	4611	7	3024
	5726		4886		3237		6195		4948		3330
	0.986552		0.974212		0.9558232		0.965617		0.931891		0.9081081
5672		0344		932		4334		6734		081	
8	4012	7	4894	12	3518	8	4142	7	5272	12	3636
	4124		5032		3552		4396		5456		3918
	0.972841		0.972575		0.9904279		0.942220		0.966275		0.9280245
9011		5167		279		2002		6598		023	
13	2942	11	2993	19	1769	13	2877	11	2950	19	1678
	3009		3058		1924		3272		3383		2038
	0.977733		0.978744		0.9194386		0.879278		0.872007		0.8233562
4663		2773		694		7286		0943		316	

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21	1785 1864 0.957618	18	2232 2317 0.963314	31	1236 1272 0.9716981	21	1749 2055 0.851094	18	2193 2511 0.873357	31	1057 1362 0.7760646
0258		631		132		8905		2282		109	
34	1038 1121 0.925958	29	1261 1264 0.997626	50	607 623 0.9743178	34	853 1121 0.760927	29	1101 1298 0.848228	50	419 566 0.7402826
9652		5823		17		7431		0431		855	
55	538 507 1.061143	47	719 697 1.031563	81	221 212 1.0424528	55	358 438 0.817351	47	491 710 0.691549	81	172 177 0.9717514
984		845		3		5982		2958		124	
89	195 179 1.089385	76	265 237 1.118143	131	37 38 0.9736842	89	105 122 0.860655	76	188 212 0.886792	131	17 19 0.8947368
475		46		105		7377		4528		421	

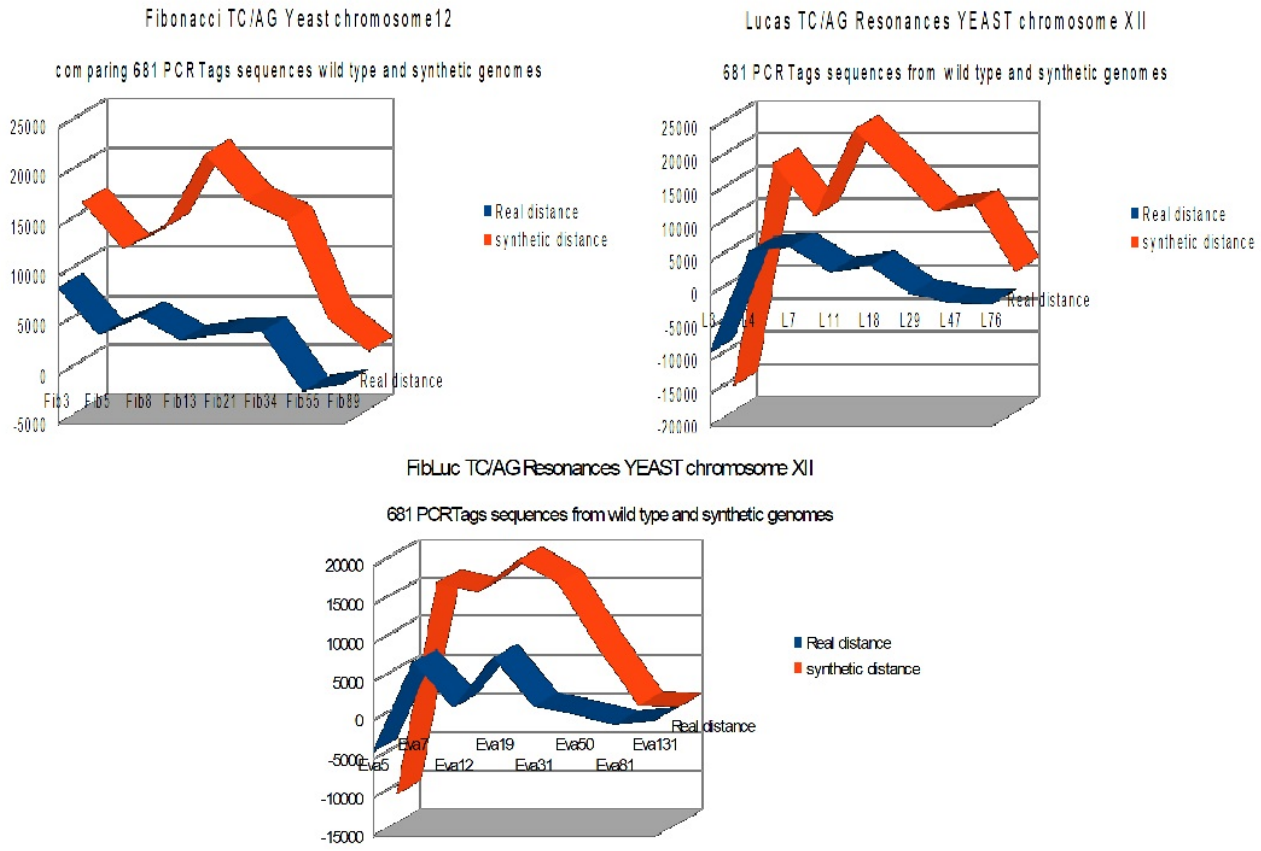

Figure 5: Comparing TC/AG Fibonacci, Lucas and FibLuc distances in real and synthetic YEAST Chromosome XII PCRTags (regular-reverse distances weighted by the length of the genome, see detailed data in suplementary materials).

V. Conclusions

In all the cases analyzed here, we find that the real genomes or chromosomes have a property of coherence, consistency and unity that our method highlights. This property disappears in almost all (ALL) studied cases of synthetic genomes or chromosomes.

Transposons: a possible explanation of global harmonics structure

In (Weiming Zhang, 2017), authors write «RATIONALE: The synthetic yeast genome, designated Sc2.0, was designed according to a set of arbitrary rules, including the elimination of transposable elements and incorporation of specific DNA elements to facilitate further genome manipulation.»

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In our article (Perez, 2010), https://www.ncbi.nlm.nih.gov/m/pubmed/20658335 we wrote:

« Why and how could this ancient code be preserved and maintained in spite of the changes and mutations during millions of years of evolution of the human genome?"

In the 1940's and 1950's, Nobel prize winner Barbara McClintock discovered a peculiar phenomenon in maize: certain regions of a chromosome moved, or transposed, to other positions. This was the discovery of TRANSPOSONS (Fedoroff, 1984): often called "jumping genes" because of their ability to "jump" to completely different regions within the chromosome and later "jump" back to their original positions. Meanwhile, "jumping genes" is a misleading term because transpositions are related to noncoding areas as well as coding areas. A particular class of transposons moves from one place to another. (Class II transposons consist of DNA sections that move directly from place to place).

Sometimes there is a palindrome-like swap of the transposon during this move.
Example, the original sequence:

5' TAAGGCTATGC 3'
3' ATTCCGATACG 5'

... Moves to another genome region and becomes reversed as follows:
5' GCATAGCCTTA 3'
3' CGTATCGGAAT 5'

We found the same process here. It joins a codon with its "mirror-codon". Perhaps DNA double strand topological reshaping processes could explain genesis of the reported facts (hairpin-like unfolding, Moebius-like ribbon, Class II transposons?)... »

These two observations about the role of transposons already partly explain the digital disharmony that we prove in this article. These famous transposons disrupt the functioning of synthetic genomes, so we delete them (!). On the contrary, we believe that these same transposons constitute a major piece of genome stability.

The creation by men of SYNTHETIC genomes leads to a paradox on which I invite you now to think about:

On the one hand, NATURAL DNA is a luxury of REDUNDANCY and SYMMETRY ...

On the other hand, SYNTHETIC DNA manipulation and synthesis technologies rely on and exploit the same luxury of REDUNDANCY and symmetries ... Thus; Sometimes the technology will try to EXPLOIT SYMMETRY and REDUNDANCY: this is the case of CRISPR technology based on DNA PALINDROMES, so on SYMMETRY and REDUNDANCY. Sometimes the technology will try to DESTROY symmetry and REDUNDANCY: Such is the case of mutations and alterations of transposons (Breuer, 2019) in order to fight against these transposons which will alter the SYNTHETIC genome. This is also the case when one tries to reduce the REDUNDANCE of the universal genetic code by reducing it from 64 to 61 codons (Fredens, 2019). By our different research on the biomathematics of DNA, we have on the contrary demonstrated that this REDUNDANCY and this symmetry contribute to the UNITY and INTEGRITY of genes, chromosomes and genomes:

When a Meta-code unifies DNA, RNA and amino acids (Perez, 2009: Perez, 2011; Perez, 2015; Perez, 2018d);

When this master code unifies the genomic and proteomic meta structures of a gene (Perez, 2000; Perez, 2017e; Perez, 2017f; Perez, 2017g; Perez 2017h);
When the multiple repetition of the same gene as DUF1220 is associated with mammalian brain properties via a kind of «FibLuc sequence» digital standing waves of its DNA (Sikela, 2006; Weiss, 2006; Parayon, 2011; Perez, 2017b);

When we prove the existence of a UNITY of Fibonacci sequences on the scale of an whole human chromosome such as chromosome4 (Perez, 2017c);

When we demonstrate how numerical proportions characterize the DNA of whole genomes of viruses, bacteria or Eucharistes (Perez 2013);

When we highlight the UNITY of the 3 billion base pairs of the entire human genome (Perez, 2010; Perez, 2017d);

When this whole human genome UNITY is destroyed by Cancer mutations (Perez, 2018a; Perez, 2018b; Perez, 2018c);

When there is an evidence that these numerical structures (Petoukhov, 2019) of the genomes, particularly SYMMETRY and REDUNDANCY, are of TOPOLOGICAL nature (Rapoport 2016). This topological unified hyper structure of whole genomes is based particularly on Fibonacci Numbers, Golden ratio (Friedman, 2018), and Klein bottle (Rapoport§Perez, 2018).

To conclude we will finally notice that the REAL genomes of bacteria analyzed obey two simultaneous numerical constraints of Phi and Phi * 2 (where Phi = 1.618 is the golden ratio and Phi * 2 = 2.618). For example, for a contiguous sequence of 21 TCAGs, we have simultaneously:

Regular (forward) 21 TCAG / 8 TC = Phi * 2

And

Reverse (backward) 21 TCAG / 13 TC = Phi.

This double strong constraint on REAL genomes almost disappears in the case of SYNTHETIC genomes.

We can not manipulate the genomes "no matter how". Thus, transposons certainly play a key role in the stability and epigenetics of genomes.

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Manipulation technologies (CRISPR) and especially of artificial creation of genomes will have to respect these laws of nature.

In (Strecker et al., 2019) by using DNA sequences referred to as transposons, or "jumping genes" (genes that can change their position within the genome), a team from MIT led by NYSCF – Robertson Stem Cell Investigator Dr. Feng Zhang has created a new version of CRISPR (called CRISPR-associated transposase, or "CAST") that can insert functional DNA sequences into the genome without making cuts, which can often lead to unintended damage.

What about for the FUTURE ? There are theoretical new background for Biology and Genetics, these tracks are MATHEMATICS (Perez § Montagnier L., 2021).

Acknowledgements

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Physiological and Molecular basis of Dormancy in Yam Tuber: A Way Forward towards Genetic Manipulation of Dormancy in Yam Tubers

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Abstract- Yam (*Dioscorea sps*) holds a key position among the staple food crops of Africa and possess better organoleptic properties compared to cassava, potato (within *Solanum spp.*) and sweet potato (*Ipomoea batatas* (L.) Lam.). Yam tubers are reported to be rich in steroidal C27 saponins and diosgenin which makes it as a crop of choice for the industrial production of pregnenolone-derived steroids. However, prolonged tuber dormancy (spanning between 120 - 180 days after physiological maturity) remains a challenge for yam improvement and production. This poses a serious threat to food security and yam industry. Furthermore, limited research efforts and low investment aggravates the slow progress in yam improvement. Deciphering the physiological and molecular factors involved in the regulation of tuber dormancy in yams will permit genetic manipulation of desired traits in yam. This requires thorough understanding of the physiological and molecular mechanisms regulating tuber dormancy in yam. The present review provides an overview on the basic hormonal biosynthetic/signaling pathways, sugar signaling pathways and the cross talk between them, which provides vital insights into mechanisms regulating yam tuber dormancy.

Keywords: *yam tuber, dormancy, physiology, molecular, hormones, sugars-metabolism.*

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Physiological and Molecular basis of Dormancy in Yam Tuber: A Way Forward towards Genetic Manipulation of Dormancy in Yam Tubers

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Abstract- Yam (*Dioscorea sps*) holds a key position among the staple food crops of Africa and possess better organoleptic properties compared to cassava, potato (within *Solanum spp.*) and sweet potato (*Ipomoea batatas* (L.) Lam.). Yam tubers are reported to be rich in steroidal C27 saponins and diosgenin which makes it as a crop of choice for the industrial production of pregnenolone-derived steroids. However, prolonged tuber dormancy (spanning between 120 - 180 days after physiological maturity) remains a challenge for yam improvement and production. This poses a serious threat to food security and yam industry. Furthermore, limited research efforts and low investment aggravates the slow progress in yam improvement. Deciphering the physiological and molecular factors involved in the regulation of tuber dormancy in yams will permit genetic manipulation of desired traits in yam. This requires thorough understanding of the physiological and molecular mechanisms regulating tuber dormancy in yam. The present review provides an overview on the basic hormonal biosynthetic/signaling pathways, sugar signaling pathways and the cross talk between them, which provides vital insights into mechanisms regulating yam tuber dormancy.

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I. Introduction

Yam (*Dioscorea spp*) is one of the oldest recorded crops eaten by human beings in many continents (1). It belongs to the monocotyledonous family, *Dioscoreaceae* and genus *Dioscorea.* It is a highly heterozygous polyploid with a basic chromosome number of 10. Most cultivars of the species Dioscorea have varying ploidy levels ranging from tetraploid to octoploid (2). Yam is a multi-species crop which has about 613 known species that produce tubers, bulbils or rhizomes. Of these, about ten are cultivated over larger area and serve as a staple food crop (3). About 50 other species are also eaten as wild-harvested staples famine food, thus this genus occupies a prominent position in global food insecurity combat, (4). *Dioscorea rotundata* and *D cayenesis* (both known as Guinnea yam) are the most popular and economically important yams in west and central Africa, where they are indigenous (5). *Dioscorea alata* has been reported as the most widely distributed species globally, because of its agronomic flexibility and high productive potential (6). Yam holds a great promise in food security, industry, medicine and overall economy in the developing countries (7). Yams are placed at fourth position among the utilized root and tuber crops globally after potatoes (*Solanum* spp.), cassava (*Manihot esculenta*) and sweet potatoes (*Ipomoea* spp.) and the second in West Africa after cassava (8, 9). It's potential as a source of food is attributed to its high levels of carbohydrates including fiber, starch and sugar, contributing about 200 dietary calories per person per day to more than 300 million people in the tropics (10). It also provides other nutritional benefits such as proteins, lipids, vitamins and minerals (11).

The growing season of yam is long (9 – 11 months) and there is a genetic variation in terms of maturity duration (early, mid and late). In the tropics, the main planting season begins in the month of February (with the planting of dormant tubers) and planting can be done till May. Some farmers plant dormant tubers during December itself. Tubers start sprouting mainly from March to May depending on the storage condition (especially the existing photothermal units), physiological age and genotypes. However, some tubers break dormancy as early as January and this early dormancy breaking is controlled majorly by the physiological age of the tuber and genotype. Harvesting of new tubers starts in the month of August (first harvest season) till December to January of the subsequent year (second/ main harvest season). Whether tubers are

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harvested in the month of August (about 180 days after planting) or November (about 270 days after planting), most of such tubers do not resume shoot growth/sprouting until about 210 days or 150 days after harvest respectively. The long waiting for the resumption of sprouting (dormancy), imposes the need for prolonged storage of seed tubers, restricts planting to once per annum, exposes up to 40% of highly-valued tubers to loss (due to pests and diseases during the compulsory storage period), exposes whole seed tubers to unplanned consumption, and these in turn contribute to scarcity of tubers especially during the planting season and consequently increased the inputs cost of yam production, (12). The cost of planting material (seed yams) alone constitutes about 40% of the total cost of yam production (13, 14). Tuber dormancy is the major cause of the prolonged inability of ware or seed tubers to sprout. Harvested tubers remain dormant; incapable of developing an internal shoot bud or external shoot bud/sprout for 150 to 210 days depending on the date of harvest, species, and growing and storage environmental conditions (15, 16). Thus, making it impossible to have more than one crop cycle per year and thereby limiting the crop production, productivity, tuber availability and the rate of genetic improvement through breeding (17, 18).

The mechanisms controlling yam tuber dormancy are not well understood, though, some studies have made valuable efforts towards elucidating the mechanisms. The objective of this review is to summarize available information on physiological mechanisms of yam tuber dormancy while adapting novel studies on other crops on genetic mechanisms of tuber dormancy. We present insights and future perspective on research for increased food security, income generation and improved livelihoods.

II. Definition of Dormancy

According to Lang *et al*, (19) dormancy is a temporary growth arrest of any plant part containing a meristem. It is an inherent plant physiological mechanism that regulates the timing of sprouting of affected plant parts (20). Dormancy period can also be defined as the period of reduced endogenous metabolic activity during which the tuber shows no intrinsic bud growth, although it retains the potential for future growth. It is highly influenced by genetic and evolutionary constituent and also affected by environmental factors such as; temperature, moisture, oxygen and CO_2 content of the storage atmosphere (21). Dormancy has been classified into three categories based on the factors that influenced growth arrest (19). These include: Endodormancy (this is a deep dormancy during which growth arrest is influenced by internal physiological and genetic factors within the meristem), Para-dormancy (this occurs due to growth arrest by physiological factors external to the meristem) and Eco-dormancy (growth is stopped by unfavorable external or environmental factors). The consequence of dormancy is severe on yam production and production system, because the duration of dormancy is very long; as much as 270 days, depending on the time of tuber harvest and definition of the start of dormancy (22).

a) Yam tuber regenerative organ and its relevance in tuber dormancy induction

During seedling development, the embryonic hypocotyl is the site of tuber induction (23). After the occurrence of adventitious root growth from the developing tuber, all other further proceeds by the diageotropic or plagiotropic lobing of the original hypocotyl bulge (24). In similar fashion, during the establishment of new plants from vine cutting, adventitious roots arise from the axillary tissue in association with the axillary bud, and not directly from the stem, while tuber induction proceeds as a result of the lobing of this axillary tissue (24, 25). Regenerative activity in whole tuber and tuber pieces begins with the production of shoot apical meristem with the associated primary nodal complex (PNC) on which induction of shoots, roots and tubers are initiated. It is on the basis of this fact that the hypocotyl of the seedling, the axillary tissues of the stem cutting, and the PNC of germinating tuber were assumed to be analogous and it is suggested that there is a common ontogeny in the tuber induction of every regenerative part of *Dioscorea* species plants (seeds, vine cutting, tuber piece) (24). This ontogeny is characterized by the production of an organ of renewed growth in the tissue subtending the stem apex. It is on this basis that organ of renewed growth in tuber has been designated as the PNC.

Burkill (26) opined that it was during the evolution of the edible *Dioscoreas* that the thickening and lobing of the ancestral rhizome gave way to a well development tuber system. With the loss of axillary buds of the rhizome, the primary thickening meristem became the site of renewed shoot growth during tuber dormancy release. This activity leads majorly to the production of a shoot and a modidfied node (PNC) which is the vestige of the ancestral rhizome and is the site of roots and tuber origin in plants. However, it has been reported that every node of *Dioscorea* including the cotyledonary node of the germinating seedling and calyptral node of the germinating tuber have the capacity to produce the vestigial rhizome-PNC (24). It seems that during phylogenic partial separation of *Dioscorea* from perennation to annual crops, the perennation potentials of the degenerated rhizome were retained in the vestigial PNC, while the new developing tuber assumes the storage role. Figure 1 shows different stages of shoot emergence from primary nodal complex.

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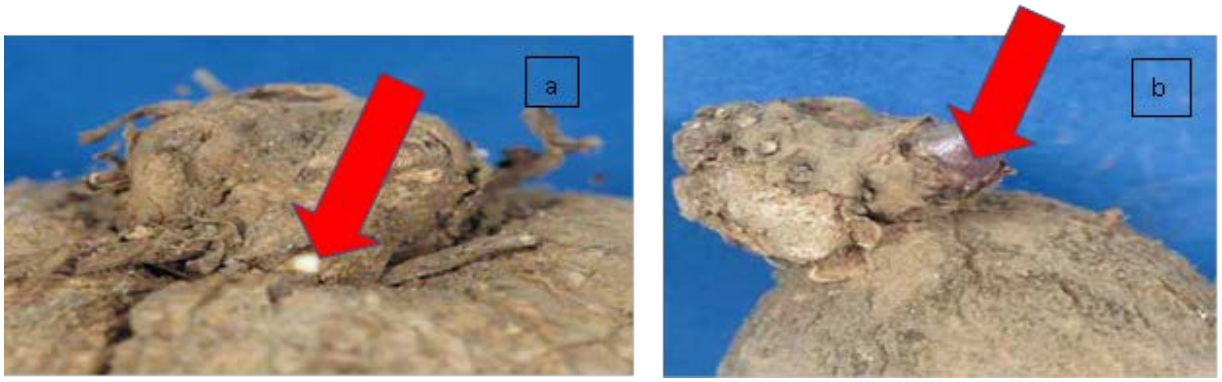

Fig. 1: The structures of primary nodal complex-PNC, showing external sprouting process at two different levels of germination locus

Structurally, PNC provides a vascular connection between the developing vine shoots and the mother tuber, the store reserve and later when the vine becomes established and start photosynthesizing and the mother tuber gives a way for new developing tuber, the vestigial PNC again connects the developing tuber and the photosynthesizing vine shoot. Primary nodal complex therefore, serves as the physical link between the new plant, its perennation and storge organs in an annual growth habit. Anatomically, there appears to be a direct meristematic continuity between the primary thickening meristem of the mother tuber, the apical meristem of new vine shoot, the PNC meristem and the primary thickening meristem of the new developing tuber being produced from PNC (24).

If the PNC or the head corm as it is also called is analogous with tuberous tissue produced by the embryonic hypocotyl and axillary tissue in vine cuttings as earlier suggested (24), then this meristematic continuity might be traced back to the rooting stem cutting and the seedling which is broken only by the mature tissue separating meristematic cells of the axillary tissue in the germinating stem cutting from vascular cambium of the stem. Thus, the yam tuber is unique is among organs of vegetative propagation because it does not contain a pre-formed bud, but has a layer of meristematic cells within the tuber cortex with the potentials of generating new plants. These cells also represent a remarkable meristematic continuity between plants of different generations with the PNC as the central organ in the continuity. Therefore, it is important that more research attention should be given to this organ through more intensive studies on the improvement yam species, particularly in the development of systems for producing cheap planting material by its multiplication which will ameliorate the impact of seed yam scarcity in yam production system and also significantly reduce long period of tuber dormancy by manipulating the meristems in the PNC at which germination also originate. Such research should also aim at building on the "tuber milking" technique of traditional yam farmers in West Africa.

b) Phases of dormancy in Yams

Dormant yam tubers are unique and in contrast to other crops such as; onion (*Allium cepa*) ococoyam (*Colocasia esculenta* L) and potatoes (*Solanum tuberosum*) in some ways. Yams do not have any internal or external apical shoot buds or sprouts, but have a layer of meristematic cells below the surface of the tuber (24). Onwueme, (27) and Wickham *et al*, (24) have shown that at the resumption of active growth, shoot apical bud formation begins in this meristematic cell layer, long before any external shoot bud/ sprout is visible on the tuber surface. Implying that the processes which culminate on the surface appearance of tuber shoot bud start long before the physical appearance of shoot bud.

According to Ile *et al,* (28) dormancy in yam tubers occurs in phases: the long phase I of dormancy (the period from tuber physiological maturity to the formation of tuber germinating meristem-TGM, which is up to 200 days). Phase II, this is the period from TGM to the initiation of foliar primordium-IFP, which is about 40 days long. Thirdly a short Phase III; the period from IFP to the physical appearance of shoot bud (ASB) on the surface of the tuber, which is only about 10 days. Shortening the period under Phase I would be useful in developing yam genotypes with reduced period of dormancy.

The two key approaches that have been suggested for solving the problem of dormancy in yam are: (1) induction of early sprouting through the prevention/inhibition of the initiation of dormancy in yam tuber such that shoot growth/sprout can resume soon after tuber formation. (2) Shortening of the duration of dormancy such that shoot growth/sprouting can resume soon after physiological maturity (180-200 days after vine emergence). From the Ile, et al., (28), it is clear that a promising approach to solving the problem of yam tuber dormancy is one that is targeted at the long phase one the TGM which also coincides with the duration of endo-dormancy that is controlled as stated earlier by internal physiological and genetic factors. This phase is

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not influenced by environmental cues, implying that they are strictly controlled by physiological/genetic factors.

c) Induction and duration of dormancy in yam tuber

There are two contrasting schools of thoughts (scenarios) on the induction/development of dormancy in yam tubers (22). Scenario A postulates that dormancy commences during tuber maturity or vine senescence/onset of the dry season and end at sprouting. In contrast, scenario B, opines that dormancy commences much earlier during the early tuber development, and ends with sprouting. This section highlights on these scenarios and their effects on: (1) the accuracy and consistency in the duration of dormancy often presented, (2) the design of research targeted at reducing yam tuber dormancy duration, (3) the timing of treatment application, and (4) the extent to which the length of the dormant period can be reduced.

i. *Scenario A*

Scenarios A is consistent with the long-standing definition; that dormancy is an adaptive mechanism developed for survival in adverse weather conditions, in this case, the dry season of the tropics. Also, in agreement with this scenario are the results of some published findings (15, 29, 30) which showed that there is a slowing down of metabolic activities in tubers with the start of the dry season. For instance, tubers that are harvested at vine senescence exhibit a reduced rate of respiration, and reduced starch and sugar metabolism. They contain high concentrations of growth-inhibiting substances, etc., with the reverse occurring at the end of dormancy/resumption of sprouting**.** It is important to note that in most of these studies, the experimental tubers were harvested at the attainment of tuber maturity or at best only a few days before this stage and the period covered is until the visible end of dormancy (sprouting). As such, the studies have provided information only on changes occurring from the defined time of harvest until sprouting.

Based on the definition in scenario A, therefore, the duration of dormancy can range from 50 to 150 days, even for the same variety, this is largely inconsistent. Some reasons for such wide variation relate to the ambiguous nature of the terms; tuber maturity and sprouting, which consequently allows the use of varied dates of tuber harvest and varied signs of sprouting. Hamadina, (22), investigated how these factors, as well as differences in species/varieties, and poorly defined/poor knowledge of environmental conditions in postharvest storage, can result in an inconsistent in duration of dormancy. The findings of this study concluded that the duration of dormancy is long and highly variable, and the variability in the duration of dormancy highlights the need for researchers to define terms clearly and describe all conditions experienced by tubers during storage and the growing season. There is no evidence that the variability in the duration of dormancy within varieties of *D. rotundata* "indigenous" to distinct agroecological zones in Nigeria, is due to inherent adaptation to their agroecology of origin/latitude of origin. Tubers, in spite of perceived differences in their agroecology of origin, tend to sprout at about the same time if grown and stored in similar environmental conditions. The growing and storage conditions/agro-ecologies are important factors affecting the duration of dormancy with the effects being as long as 20 days. Based on the effects of exogenous PGRs on the duration of whole tuber dormancy as well as the effects of physical and environmental factors, it is clear that whole tuber dormancy, in the context of scenario A, can be shortened only by about 30 days using agronomic approach.

ii. *Scenario B*

Some researchers have hypothesized that tuber dormancy does not begin when tubers reach agronomic maturity or leaf/vine senescence, But rather much earlier during early tuber development. This school of thought holds that dormancy begins sometime during tuber development and ends before sprouting (15, 31). This second group suggests that there is a 'true" dormancy period (endo-dormancy) that starts during tuber development and ends well before sprouting, being marked by the onset of activity in the meristematic region that leads to the formation of the internal shoot bud (21, 27). Only a few studies have been carried out within the context of this school of thought. Although the reason for this is not clear, it is supposed that scenario B has been unattractive, probably due to the fact that it implies that actively growing and developing tubers exhibit dormancy and sampling growing underground tubers for analysis may be a tedious task. Another reason may be because it implies that yam tuber dormancy (observed in whole harvested tubers) may not arise simply due to the effects of adaptation to a prevalent or impending adverse environmental condition (such as the advent of cold periods in temperate regions and the dry season in tropical regions.) and thereby highlighting the fact that genetics is much involved in the mechanisms regulating tuber dormancy. The consequence of limited research in this area implies that the factors that affect the initiation and duration of dormancy are not clearly understood and evidence that elucidates its control mechanism(s) is more than eco-physiological factors as suggested by scenario A.

Again, Hamadina (22) findings concluded that; Dormancy commences much earlier, during tuber initiation and development, rather than later. The duration of this dormancy is much longer than its estimation under Scenario A and covers a larger part if not all of the period of dormancy. The difference in the duration of dormancy/timing of sprouting among landraces of *D. rotundata* is not related simply to

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provenance/adaptation to the agroecology of origin, i.e., durations of the dry or rainy season, but instead the duration of dormancy varied, depending on genotype, growing and storage conditions. Inductive environmental and endogenous factors, such as air temperature, photoperiod, relative humidity, and exogenously applied/endogenous PGRs, etc., can slightly shorten the duration of dormancy. From this school of thought, yam tuber dormancy seems to be regulated more by genetic factors than environmental factors. Therefore, this scenario tends to be more wholistic in viewing of yam tuber dormancy induction. However, its deficiency lies on the fact that even Hamadina (22) established that some of tuber initiating and development phytohormones (endogenous PGRs) also have dormancy inducive effects; inhibiting sprouting even on physiologically mature tuber and these substances are in their peak concentrations during tuber development and gradually decrease, even after the tuber development has come to an end and tubers attain maturity. This implies that phytohormones involve in tuber initiation, growth and development are also part of the hormones involve in tuber dormancy induction and maintenance, this might be the tuber internal mechanism of ensuring that growing tuber cannot initiated sprouting process which will limit its growth potential and as well affect it food quality. The pictorial summary of the postulations of the two scenarios of dormancy induction and duration n yam tuber and actual empirical observation is presented in figure 2 below.

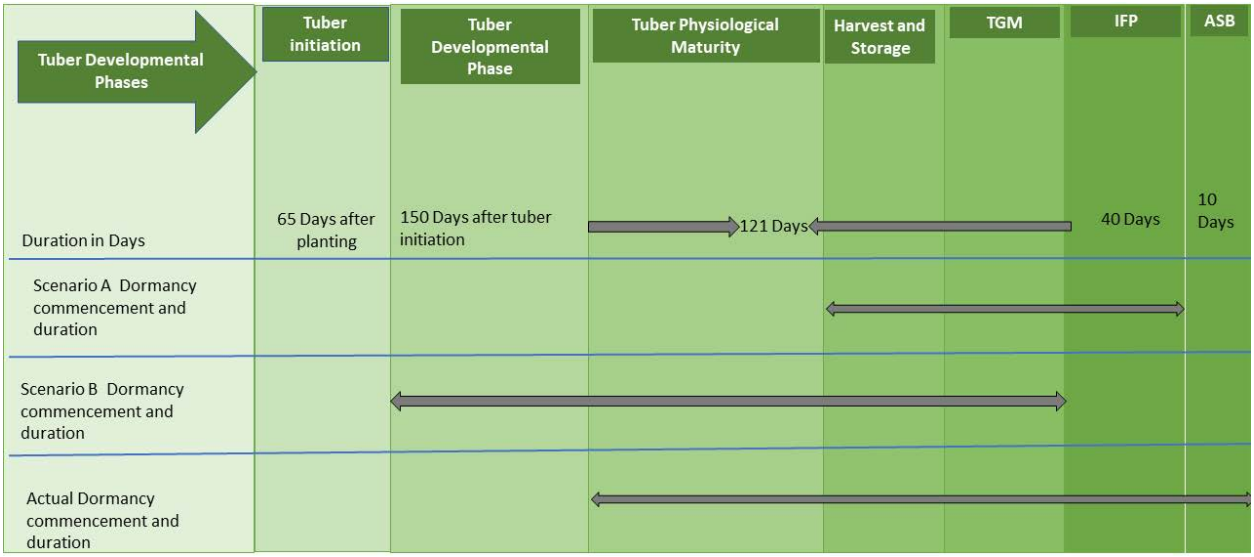

Fig. 2: Diagrammatical representation of yam tuber developmental phenology. Showing the proposed tuber dormancy induction and duration according the two lines of hypotheses, and the actual dormancy induction phenophase and duration based on empirical observation

It is necessary to find the agronomical ideal time of commencement and duration of tuber dormancy in order to design research towards its efficient management. This will lead to striking a balance between the two schools of thought, even though, each of them has its merit, but fact remains that an ideal definition lies somewhere in the middle. As already stated here, tuber initiating and development hormones are also dormancy inducing or sprouting inhibiting substances, it implies that tubers are at early developmental stages are designed to be dormant, therefore, tubers are produced dormant and the production hormonal machinery helps to maintain that dormancy during early development to ensure optimum tuber development, food quality and shelf life. Hence, in designing research targeted at reducing the long tuber dormancy duration, this growth and developmental stages should be excluded, because tilting the concentrations of those tuber developmental PGRs during early tuber development stage in order to induce sprouting at such developmental stage might have some serious negative implications on tuber economic yield, food quality and shelf life.

In view this, the question of when is agronomically ideal commencement of dormancy induction needs to be answered, to clearly define what part of this long yam tuber dormancy period has constitute a constraint to yam production, productivity and genetic improvement. And this, an average yam researcher and producer will agree that it is from physiological maturity or onset of senescence. If tubers can be made to be able to initiate the processes of sprouting from physiological maturity, a reasonable dormancy period reduction would have been achieved. On the other hand, contrary to the position of first school of thought (scenario A) which seems to be inferring that yam tuber dormancy is absolutely controlled by environmental and eco-physiological factors, scenario B

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position which concluded that genetics and storage condition instead of only environmental and eco-physiological factors are regulating the duration of endo-dormancy is more accurate from the findings of Hamadina (22). Hence, it could be concluded that accurate definition of the commencement of dormancy induction is when supply of metabolites and photosynthates from source sites (photosynthesizing vegetative parts) to sink (tuber; the storage organ) is terminated. And this always coincide with the commencement of leaves/vines senescence, indicating that tuber filling has stop, thus marking the end of tuber growth and development. A growing tuber, even though it lacks differentiated meristematic cells and is incapable of sprouting, cannot be described as a dormant tuber either as scenario B postulated because the hormonal machinery involve in tuber growth and development are also sprout growth inhibitors and as such will not permit sprouting of growing tuber.

d) Anatomical basis of yam tuber dormancy

The cellular anatomical structure of yam tuber is quite striking and it influences every physiological process in the tuber. Studies on cellular anatomical structure of yam tuber have revealed distinct fragmented pattern which was linked to tubers ability to germinate and grow new plants (32). Generally, in cellular anatomic structure of yam tuber there are five major identifiable regions and additional one region conspicuous only in *D. alata* (33). Each of these anatomical regions performs one or more cellular physiological functions that drive the whole plant phenotype including determination of yam tuber dormancy. The anatomical structure is organized in the following layers: (1) Cork layers comprising of primary and secondary cork layers. It is an average of 5.05 layers of suberized corky cells tangentially elongated and disposed in radial series, the inner layers being often compacted against a basic cambial layer. It performs mainly the functions of mechanical protection and providing firm frame, as well as regulating respiratory and hydric economy of food stores. (2) Cambium layer; this is located at the outer cortex and sub-apical meristem and it performs the function of sensor cells by relaying information through and fro from the inner cortex to the outer region. (3) Cortical parenchyma; this layer contains more or less tangentially elongated cells with secretory and raphide cells. It serves mainly as the store house, packs of grana and starch grains. (4) Procambium; This is an area of sheath and lengthening cells like layer. It is involved in the tissue conductivity (24).

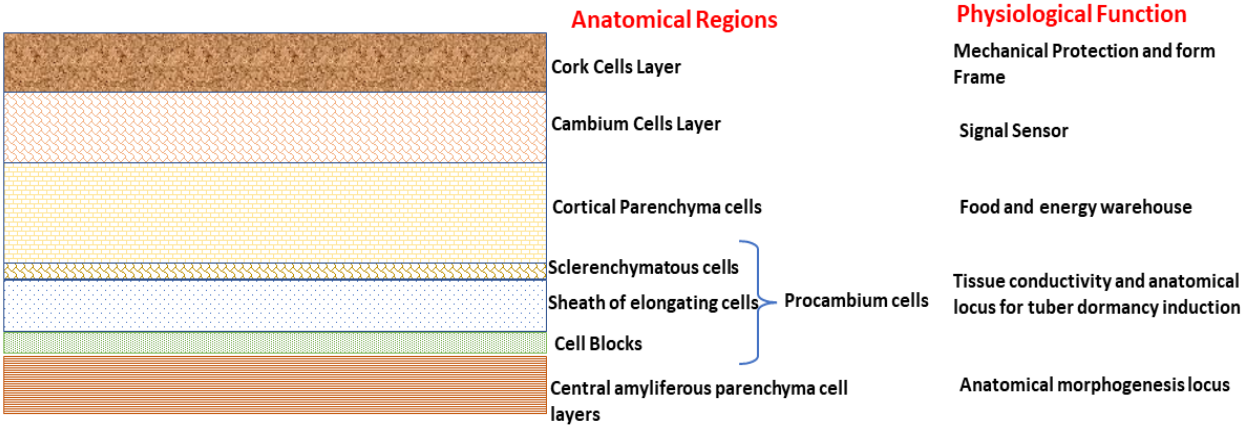

Fig. 3: Hypothetical anatomical structure of dormant yam tuber. Showing different anatomical cell layers and their physiological functions, revealing cell blocks as the anatomical locus for dormancy induction in yam tuber

Procambium layer also contains individual cell blocks that function as organizing pole structure and determine growth and morphogenesis. they can be compared to the undifferentiated embryo of some conventional seeds, and are located in the main part of tuber cellular anatomic structure of mature tuber between harvest and germination, where they maintain a kind of embryonic dormancy and also determine other organogenetic processes(24). In fact, this particular layer has been described as a centre for species diversity and site for growth initiation and multiplication in any tuber. Hence, the individual cell blocks in procambium can be described as cellular anatomical locus for dormancy trait. According to Wickham, et al (24) the general presence of generative cell blocks offers histological basis for the structural and dynamic analysis of the tuber multiplication.

In *D. alata* procambium layer equally contains thick layer of distinct sclerenchymatous cells which is the main centre of specie specific diversity. According to Boureau, (34) it contains something like the “transfusion tissue” which play the role of water conservation in the tubers of *D. alata,* and their cell wall punctuation and the vascular bundles vicinity are good for its function. It was also suggested to be the factor that conferred wide ecological adaptation and dispersion potentials on *D.*

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alata (35). (5) Central amyliferous parenchyma is a layer of vascular bundles of cells growing in size outwards, but often blocked by individual cell blocks in procambium layer above it. It contains calcium oxalate and tannin cells and also play roles in osmotic pressure balance (33), and modifies the respiratory quotient and detoxify the system. It is speculated to be linked with a high metabolic activity, which is indicated by the multiplication of raphides in the growing zones (36). Figure 3 above shows the schematic diagram of anatomic structure of dormant tuber, depicting the anatomical mechanism of dormancy induction in yam

III. Regulation of Dormancy in Yam Tubers

Yam tubers enter into dormancy enduring tuber bulking and vine senescing. The longevity of dormancy depends on levels of phytohormones, the crosstalk between them, intricate genetic regulatory networks, as well as environmental cues (37). For decades, some molecular and physiological surveys have revealed that different hormonal pathways regulate different aspect of tuber development (38-41). Though, these studies were conducted on other crops, however, an evolutionary survey has revealed strong similarities between *Arabidopsis*, tomato and potato in hormonal dynamism, crosstalk, signaling pathways and the networks regulating seed and tuber dormancy, indicating conserved evolutionary processes across a wide spectrum of plants (38). Since potato and yam tubers share very close physiological and morphogenetical communalities, it is believed that the molecular and physiological machineries regulating dormancy in the two crops will be similar, some specie specific different notwithstanding. Therefore, due to lack of information on molecular and physiological mechanisms regulating yam tuber dormancy, information on potato tuber will be adapted in discussing yam tuber dormancy here. It is now clear that abscisic acid (ABA) gibberellins (GAs), auxins, and to lesser extent cytokinins (CKs) and ethylene (ET), are the main phytohormones that play key roles in molecular and physiological regulation of dormancy in both conventional seeds, tubers, rhizomes and bulbs (37, 42-46) While crosstalk between the main stream regulatory hormones signal networks and some other phytohormones like strigolactones, brassino-steriods jasmonic acid salycylic acid also play some roles (47). There is also sugar metabolism and the signaling crosstalk with phytohormones in dormancy regulation in several crops(48-50). However, in this review due to want of space, discussion will be limited to three main phytohormones (ABA, Auxins, GAs) and Sugars metabolism and the crosstalk between sugar signaling pathways and hormones regulatory networks.

a) Abscisic acid mediated regulation of dormancy

In conventional seeds, at maturation the embryo is kept in a quiescent state in which all nutrients are stored without any mobilization and no cell division or elongation takes place. Hence, germination-promoting genes are not activated, this is because the radicle does not penetrate the testa and endosperm, where it can access sugar for energy and nutrients required to initiate growth processes (51). Similarly, in non-conventional seed like yam tuber, similar phenomenon also takes place, for instance in mature dormant tuber the anatomical structure presented in (fig 2) above revealed that at maturity; the procambium region which is responsible for growth, morphogenesis and tissues conductivity is separated from central amyliferous parenchyma layer (the food and nutrient warehouse of tuber) by a layer of cell blocks and as long as this block is maintained, dormancy is maintained and germination is blocked. Because for the processes of germination to be initiated the procambium cells must gain access to the amyliferous parenchyma layer to transport nutrients and sugar that will provide the required energy to initiate the processes at the upper region. Therefore, procambium, cell blocks and central amyliferous parenchyma can be likened to be radicle, tasta and endosperm of tuber seed. It has been demonstrated that the chromatin structure determines the expression of genes and thereby regulates several developmental processes (51). Many genes associated with chromatin remodeling have been reported to regulate also seed dormancy and germination (37, 52-54). Evidence indicates that abscisic acid (ABA) is involve in chromatin remodeling (55). For example, the histone methyltransferase gene *KYP/SUVH4* is repressed by ABA(53), while histone acetyltransferase gene *HvGNAT/MYST* is induced by ABA (56), and as expected epigenetic regulating genes *HUB1* and *RDO2* are up-regulated during seed dormancy induction. This is because during dormancy the cell is not undergoing cell division and the chromosomes are tightly packed by histone proteins, therefore, activation of histone proteins will likely be repressed by any factor that positively influence dormancy induction and maintenance such as ABA and other phytohormones.

ABA is derived from epoxycarotenoid cleavage and is one of the most important plant hormones, with most versatile roles in various physiological functions of plants such as; transpiration, dormancy induction, maintenance and germination and improved resistance to extreme environmental stress during plant development (57-59). Maternal ABA has been reported to play a key role in embryo morphogenesis and desiccation, stomatal movement, synthesis of stress proteins and metabolites and seed maturation in tobacco and *Arabidopsis* (41, 60, 61). However, ABA is also *de novo* synthesized in embryo and testa and accumulates during embryo development, seed

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maturation, and facilitates late seed maturation processes, synthesis of storage proteins to prevent seed abortion, induce primary dormancy and as well as allows successful germination of the successive seedling (62). Kanno et al (63) demonstrated that ABA synthesized in both maternal and zygotic tissues during seed development, and maternal ABA can be translocated to the embryos and induced seed dormancy. ABA deficient mutants of maize (*Zee Mays*), *Arabidopsis* and tomato (*Solanum lycopersicum*), rice (*Oryza sativa*) and *Nicotiana tobacco* lost their dormancy potential and resulted in precocious seed germination and viviparity (64-67). Liu, et al (66) further demonstrated that exogenous application of ABA in three rice cultivars positively correlated with their seed dormancy. Similarly, results of analysis of endogenous ABA content in vegetive reproductive organs have revealed that ABA plays key role in bulbs, root and tuber dormancy induction and maintenance (37, 44, 68). For instance, combined analysis of transcriptome and targeted metabolome has revealed that in lily bulbs, *AB13* and *AB15* which are both necessary precursors for ABA induced *AtWRKY2* expression which reduced dormancy duration, while *AtWRKY2* knockout mutant bulbs exhibited increased dormancy duration under ABA high content (69). The *AtWRKY2* expression induction by *AB13* and *AB15* which lead to bulbs dormancy duration reduction may be as a result of rate limiting feedback mechanism of these ABA precursors that might negatively regulated some of signal pathways which were corrected by *AtWRKY2* knockout and exogenous ABA treatment. ABA has also been implicated in dormancy induction and maintenance in barley seed (70). Analysis of ABA deficient or insensitive mutants of various barley species that exhibit short dormancy duration or pre-harvest sprouting has provided strong evidence that ABA is involve in dormancy initiation during barley seed development (71, 72). The growth inhibitory activity of ABA has also been reported in standard ABA bioassays of crops such as the *Avena cepa,* wheat coleoptile and lettuce hypocotyl (73, 74).

Other studies have shown that *NAC* family is involved in regulating multiple hormones signaling pathways some of which negatively influence ABA dormancy induction. It has been reported that *GhNAC83* affects the dormancy of gladiolus bulbs by negatively regulating ABA signal transduction and cytokinin biosynthesis (75). Also, Kim et al, (76) reported that ABA controls dormancy and bulb formation in lily plants, whereas, fluridone (ABA inhibitor) prevents dormancy induction when both of them were separately applied exogenously. Similarly, it has been demonstrated that ABA controls dormancy induction in onion bulbs, but not involve in onion bulb formation as decreased level of ABA by fluridone application did not affect the formation bulbs scales (46), but reduced dormancy duration. Furthermore, Alamar, et al, (44) concluded that ABA and its metabolites (phasic acid) induced and prolonged onion bulb dormancy under ethylene supplementation. In potato tuber, ABA has been reported to be involved in regulation of dormancy induction and wound healing (77, 78). ABA content was observed to be highest immediately after harvest when meristem dormancy is deepest, and gradually fall during storage as dormancy weakens (77). Similarly, it has been demonstrated that ABA play key role in mediating potato dormancy which has been well characterized in meristematic tissue and it is shown that ABA accumulation reaches maximum during tuber and dormancy induction and declining of ABA content was shown to be the determinant factor in potato tuber dormancy breaking (79-81). Recently, Tosetti et al,(43) also demonstrated that parenchymatic tissue ABA content reached maximum at onset of vine senescing which coincides with the onset of dormancy induction, but was rapidly decreased by continuous ethylene treatment which led to earlier dormancy breaking. Implying that ethylene antagonistically prevents the ABA dormancy induction in potato tuber.

Most of the studies on yam tuber hormonal control of dormancy have concentrated mainly on abscisic acid (ABA) related analogues compounds such as phenolic growth inhibitors and in particular batatasins. Batatasins belong to the phenolic class stillbenoids. They occur naturally in many plant species exhibiting dormancy. In *Dioscorea,* they have been isolated in *D. alata, D. cayenensis,* and *D. opposite* (73, 82). They are more concentrated in the peel, (the region closest to the meristematic layer where sprouts originate) than in the pulp. By isolating these compounds over time, it has been revealed that the concentration of batatasins increased from 150 days after planting, attaining a maximum at tuber maturity, when tubers are declared dormant (83), then declined gradually until sprouting (73, 82, 83). Exogenous application of batatasins I, II, III, IV, and V have inhibited the growth of shoot and buds in potato and other plants, delayed the appearance of shoot and buds in some yam spp., for example*, D. alata, D. cayenensis,* and *D. esculenta* by about 15 days (83-85). In *D.alata*, the true dormant period (endodormancy) has been estimated to be about 220 days which begins from the onset of tuber induction to appearance of tuber germination meristems (TGM) and is not affected by PGRs. But in *D. alata* specie, seed tuber duration of this long endodormancy period has been drastically shortened by fluridone (an ABA biosynthesis inhibitor) to extent that still growing tuber started showing anatomical signs of germination (86). Also, Hamadina and Craufurd (87) reported that the level of phenolic compounds increased in *D. rotundata* is higher in developing tubers than at tuber maturity/ vine senescence, implying that this compound is also involved in the tuber development.

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i. *Role of ABA biosynthetic genes in inducing dormancy*

ABA is synthesized from epoxycarotenoids cleavage (zeaxanthin, violaxanthin, and neoxanthin) which is often initiated in the chloroplast and proceed to cytoplasm, its biosynthesis, signaling and degradation genes have been reported to play important roles in dormancy induction, maintenance and release (42). There are three groups of genes that have reported to be involved in the stepwise ABA biosynthesis which include; *Zeaxanthin Epoxidation (Zep), Oxidative Cleavage of 9-Cis-Epoxxycarotenoids (Nced)* and *Abscic Aldehyde Oxidation (Aao)*(88). The first step of ABA biosynthesis is the conversion of zeaxanthin to all-*trans-violaxanthin*, catalyzed by the zeaxanthin epoxidase (*ZEP*)(89). Antheraxanthin is formed as an intermediate product of the reaction. Then, the conversion of all-trans-volaxanthin to 9-cis-violaxanthin or 9-cis-neoxanthin mediated by yet to be identified enzymes(90). The oxidative cleavage of 9-cis-violaxanthin and or/ 9-cis-neoxanthin is catalyzed by 9-cis-epoxy carotenoid (*NECD*) which leads to the formation of a C_{15} product, xanthoxin and a C_{25} metabolite in a reaction which has been described as rate limiting step and *NCED* is the key enzyme in the biosynthetic pathway(89). The *NCED* family of genes comprises of *NCED*1-9. The next ABA biosynthetic step takes place in cystol in which Xanthoxin formed earlier is converted to ABA through two enzymatic reactions. In the first reaction, xanthoxin is converted to abscisic acid aldehyde by an enzyme belonging to short-chain dehydrogenase/reductase (SDR) family, the gene responsible for these reactions has been identified as *ABSCISIC ACID INSENSITIVE* (ABI) which comprises of *ABI2, AB13, ABI4* and *ABI5*. These are the signaling genes that are responsible for ABA dormancy induction and maintenance capability, however, among them, *AB13* is outstanding in this function as expression of its transcripts has been reported to be highest in dormant seed and decrease after germination (88). The second Xanthoxin conversion reaction and final step of ABA biosynthesis is the oxidation of abscisic aldehyde to ABA, catalyzed by an abscisic aldehyde oxidase (*AAO*) (91). ABA biosynthetic pathway is important because it reveals different genes involved the ABA biosynthesis and provides various points of the biosynthetic pathway that can be manipulated to effectively reduce the effects of ABA on dormancy maintenance through genetic engineering. For instance, the *ZEP*/ABA were first to be identified in *Arabidopsis thaliana* and *Nicotiana plimbaginifolia* (42, 89, 92).

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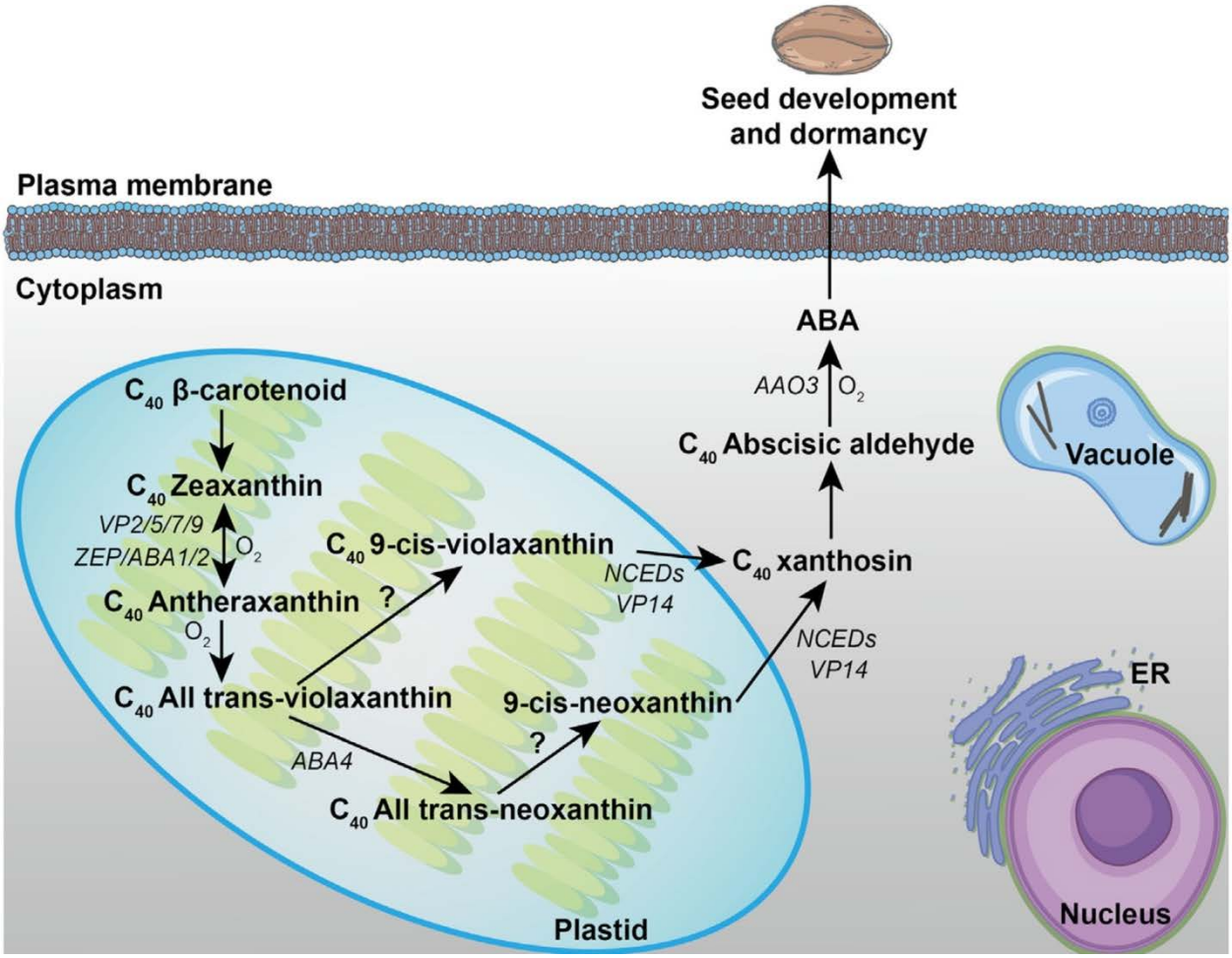

Fig. 4: Dormancy regulation and ABA biosynthesis through the carotenoid pathway starting from β-carotene in the plastid and ending with Abscisic aldehyde conversion to ABA in the cytoplasm, indicating the major genes and enzymes responsible for each conversion reaction. The arrows with question marks indicate the reactions which conversion factor are not yet identified *Source: Ali et al, (2021)*

Their ABA deficient mutants with (aba1/aba2) exhibited impaired oxidation of zeaxanthin into antheraxanthin and violaxanthin which is the initial step of ABA biosynthesis (Fig3). Similarly, n rice, viviparous mutant genotype was identified to exhibit viviparous germination as a result of defect in the oxidation of zeaxanthin during ABA synthesis (93). In maize, different auxotrophic mutants (*vp2, vp5, vp7 and vp9*) have been identified through genetic screening and they exhibit defects in zeaxanthin epoxidase activity and block the early steps of carotenoid biosynthesis (42). Whereas, overexpression of maize *VP1* in wheat induced increased duration of seed dormancy and prevented pre-harvest sprouting (92). All these provided evidence that the oxidation of zeaxanthin is an important, and a conservative phase in the ABA synthesis in plants. Another important gene and stage of ABA biosynthesis is the *NCEDs* and the conversion of all-trans-violaxanthin to 9-cis-violaxanthin or 9-cis-neoxanthin. *NCED9* was first cloned from maize mutant *VP14,* the *VP14* mutant which exhibited defect in the oxidation of all-trans-violaxanthin to 9-cis-violaxanthin or 9-cis-neoxanthin and exhibited reduced ABA content in matured seed and consequently has reduced dormancy duration (42). In *Arabidopsis NCED2, NCED3, NCED5, NCED6,* and *NCED9* have been identified as the homologs of *VP14* participating in the rate-limiting step of ABA biosynthesis (94). Also, the *PvNCED1. LeNCED1* and *BdNCED1* were identified in bean, tomato, and *Brachypodium distachyon*, respectively and they showed important roles in ABA biosynthesis and seed development and dormancy induction (95). These studies have provided evidence that the oxidative cleavage of xanthophylls is the main step during ABA biosynthesis regulation of seed development and dormancy. Mutants *facca* and *sittens*, which are defective in abscisic aldehyde oxidatively conversion into ABA were first identified in tomato, and later abscisic aldehyde oxidase3 (*AAO3*) was identified in *Arabidopsis* which functions in the last steps of ABA biosynthesis in seed and its expression was also observed in the embryo vascular tissues during mid and late maturation phases (89, 91). Figure 4

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shows ABA biosynthesis from carotenoid pathway and dormancy induction mechanism.

ii. *Roles ABA signaling networks in dormancy regulation*

ABA signaling networks also play vital roles in dormancy induction, maintenance and releasing. The core ABA signaling involve in dormancy induction is mediated by pyrabactin resistance proteins/PYR-like proteins/regulatory components of ABA receptor (*PYR/PYL/RCAR*), phosphatase 2C (*PP2C*), SNF1-related protein Kinase 2 (*SnRK2*), and abscisic acid responsive elements-binding factors (*AREB*) and basic leucine zipper(*bZIP*) transcription factors (96-98). In *Arabidopsis*, ABA signaling genes are also implicated in seed dormancy regulation, for instance, *ABA sensitive* 1 (*ABI1*) encodes *PP2C* phosphatase, and negatively regulated ABA signaling (99). It has been reported that *ABI1* loss of function mutant (*abi1*) exhibited reduced dormancy duration and better seed germination in the presence of optimum ABA content level(100), this could be attributed to lack of *ABI1* function in the system and thereby confirm that *ABI1* is required for ABA-mediated dormancy induction and that ABA signaling regulatory genes also play key roles. Other *PP2C* phosphatase, *HONSU*(HON), also represses ABA signaling specifically in seed, *HON* expression is associated with both, dormancy induction and releasing (101), it seems to act in rate-limiting manner that enables it to induce both dormancy and dormancy release. Among the *ABI* genes, *ABI3* is the most influential in dormancy induction, and it is expressed in the growing seeds, where it regulates the accumulation of chlorophyll, anthocyanins, and storage proteins together with two other seed-related regulators such as; *FUSCA 3 (FUS3)* and *leafy cotyledon 1 (LEC1)* (95, 102). Loss of function mutant of *ABI3 (abi3)* has been reported to show no dormancy at all and immature seeds are able to germinate (103). *ABI3* is regulated by *WRKY* DNA-binding protein 41 (*WRKY41*), during seed primary dormancy induction *WRYK41* binds directly to *ABI3* promoter and to induce its expression (104). The ABA biosynthetic pathway offers opportunity to understand an active ABA pool during plant development that is controlled by various homologous genes. Identification of cofactors of the enzymatic reactions in the ABA biosynthetic pathway would be helpful in understanding of the complete networks of ABA synthesis and offer opportunity for effective dormancy duration manipulation in long duration dormant crop like yam tuber through genetic engineering.

b) Role of Gibberellic Acid (GA) in dormancy regulation

Gibberellins are phytohormones that comprise of a large family of diterpenoids which possess tetracyclic *ent*-gibberellane carbon skeletal structure arranged in either four or five ring systems, where the variable fifth ring is a lactone (105). GA promotes seed dormancy release and germination, and its biosynthesis and responses are highly coordinated during dormancy releasing process (106). Activation of GA-responsive genes induces cell wall- remodeling enzymes, such as, as endo-β-mannase, xyloglucan endotransglycolase, expansin, and β-1,3-mannase. Their activity leads to the weakening of the embryo-surrounding layers, and thereby stimulate growth in the embryo (92). The complex regulatory events in GA signaling pathway include cross talk with other hormones, environmental signals and regulation of genes involved in promoting cell elongation and division (107). Accumulation of GA in the radicle of embryo is accompanied by a reduction in ABA content suggesting GA and ABA antagonistic roles in dormancy regulation (108).

i. *GA metabolism and dormancy regulation*

The biosynthetic pathway of GAs starts from geranyl-geranyl diphosphate (GGDP) through pentenyl diphosphate (*IPP*), which is the 5-carbon building block for all terpenoid /isoprenoid compounds (109). Figure 5a below shows the GAs biosynthetic pathway, indicating the stepwise molecular processes, while 5b indicates the perception of environmental signals by the GAs biosynthetic pathway and crosstalk with other protein molecules in dormancy regulation. The basic isoprenoid unit *IPP* is generated via two pathways: mevalomic acid (MVA) pathway in cytoplasm and methyl erythrito phosphate (MEP) pathway in plastids (105, 110). The full route is divided into three stages according to their subcellular compartment and enzymes involved. The two-step conversion of GGDP to *ent*-Kaurene is catalyzed by *ent*-copaly di-phosphate synthase (*CPS*) and *ent*-Kaurene synthase (*KS)* (105). Both enzymes have been reported to be encoded by single locus in *Arabidopsis* (GA1 and GA2) respectively and in rice (*OsCPS1* and *OsKS*1) respectively, while in pumpkin (*cucucurbita maxima* L.) only one gene coding for *KS* has been identified (111-113). Conversion of *ent*-Kaurene into GA_{12}-aldehyde is catalyzed by the KO and KAO enzymes. In *Arabidopsis*, one single *KO* gene (*GA3*) and two KAO genes (*KAO1* and *KAO2*) have been identified and functionally characterized, where their loss of function mutant (*ga3*) exhibited growth delay in germination and defective growth phenotype (114). In rice, mutations in the *OsKO2* resulted in severe GA-deficiency, prolong dormancy and dwarfism (115), whereas, in maize (*zea mays)* two putative KO genes have been identified and *CYP701A26* was characterized to exhibit *ent*-Kaurene oxidase activity which led to increase in the accumulation bioactive GAs and consequently resulted in reduction of dormancy duration, while in barley, one single *KAO* gene which exhibited similar trit phenotype was found (105, 116).

Contrary to previous observations, two *KAO* genes have been identified in Pea, but one exhibits previously observed phenotype, while the second was

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constitutively expressed in developing seeds (117), suggesting that GAs may be playing a role in pea seed development. The oxidization of GA_{12}-aldehyde to GA_{12} which is primary precursor of bioactive GAs in plant is catalyzed by dioxogenase (GA 7-oxidase, (GA7ox) (105). The conversion of $GA_{53/}$ GA_{12} to GA_1/GA_4 is executed through two parallel routes: early 13-hydroxylation and non-13-hyroxylation pathways. In rice, the early 13-hyroxylation pathway involves the activity of 13-hydroxylases which are coded by *CYP714b1* and *CYP714B2* genes (118), and it has been suggested that the early step could be rate limiting feedback mechanism that checks the growth inducing action of GAs as its over expression resulted GA-deactivation consequently to delay in seed germination and reduced growth in shoot.

Transformations of GA_1/GA_4 to GA_{20}/GA_9 is catalyzed by two soluble 2-oxoglutarate-dependent dioxygenases (2ODDS) known as GA20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) (119, 120). Expression of these genes have resulted to reduced dormancy duration phenotype and were also observed to be constitutively expressed in the regions of active growth such as shoot, root and flower initiating organs in several crops (105, 121-124). GA signaling is known to be regulated by a group of repressors called *DELLA* proteins, including repressor of ga1-3 (*RGA*), GA-INSENSITIVE (GA1) and repressor of ga1-3-like1/2/3 (*RGL*1/2/3) (106). Among these, RGL2 seems to be the major *DELLA* factor involved in repression of GAs activity and seed germination. Studies have shown that *RGL2* stimulates ABA biosynthesis by inducing the expression of *XERICO* and *ABI5*, whereas, ABA enhances *RGL2* expression (125, 126), indicating that *RGL2* mediates the interaction between GA and ABA in dormancy regulation.

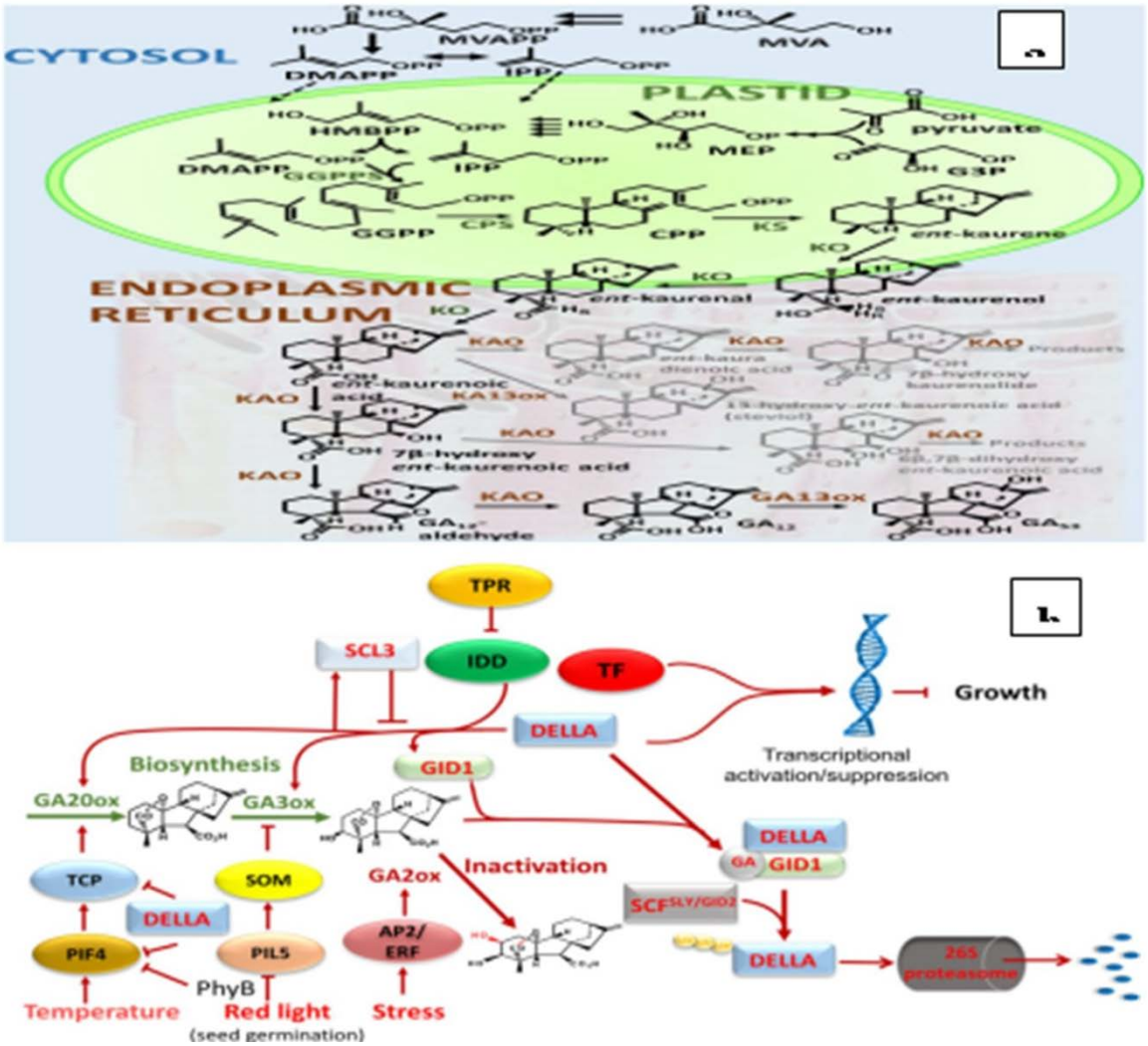

Fig. 5 (a): GA biosynthetic pathway indicating reactions in the cytosol, plastid and endoplasmic reticulum in dormancy breaking seed. (b) GA biosynthesis regulation indicating the inactivation action of *DELLA* proteins and the crosstalk between environmental signals and GA signal transduction pathway that maintain GA homeostasis. The arrows show actions that are successfully executed, while arrow bars show actions that are blocked. *Source: Sponsel and Hedden, 2010*

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c) Role of Auxins in dormancy regulation

Auxin is an exceptional plant hormone, it is the only that controls its own long-distance transport system, and it affects all aspects of plant life, including embryo development, cell division and differentiation, general plant architecture and orientation in space, stress responses and tuber wound healing (47, 127). Indole-3-acetic acid (IAA) is the common natural occurring form of auxins in plants, but other natural (4-chloroindole-3-acetic acid, 4-Cl--IAA; phenylacetic; PAA) and synthetic (1-naphthaleneacetic acid, NAA; 2,4-dichlorophenoxyacetic acid, 2, 4-D) etc., also exist (128).

Auxin alone was not previously considered an important regulator of seed dormancy and germination. Earlier studies have suggested that exogenous auxin can suppress seed germination under saline stress conditions (129), implying that auxin plays regulatory role in seed germination in response to environmental cues. It has been reported that IAA could inhibit Pre-harvest sprouting in wheat through ABA repression of embryonic axis elongation by stimulating auxin signaling (130, 131). Another study suggested that after-ripening treatment-mediated dormancy release is correlated with decreased seed sensitivity to auxin (132), suggesting that the after-ripening might have deactivated auxin biosynthetic pathway or signaling network.

The exact mechanism underlying auxin action on seed dormancy is largely unknown until recently. Genetic data has demonstrated that auxin regulates seed dormancy via the ABA signaling pathway. Auxin responsive factors (*ARFs*); specifically, *ARF10* and *ARF16* have been reported to indirectly activate *ABI3* transcription and *ABI3* is the key dormancy inducing ABA biosynthetic transcription factor, therefore, activating *ABI3* will result in increased ABA accumulation and consequently dormancy induction (122). Furthermore, another study has revealed that seeds of *Arabidopsis abi4* and *abi5* mutants are insensitive to auxin treatment during germination indicating that ABI4 and ABI5 are important regulators of auxin-mediated dormancy induction and maintenance (133, 134). The synergistic effects of IAA and ABA on seed dormancy was also demonstrated by the loss of function of mutant *abi3-1* which exhibited reduced dormancy phenotype in the presence of optimum IAA concentration (135). Similarly, intense seed dormancy and ABA hypersensitivity of the *iaaM-OX* line were compromised in the *iaaM-OX*/*abi3* double mutant confirming that the synergistic effect of IAA/*ABI3* is required for auxin seed dormancy induction (136). Furthermore, seed dormancy and ABA sensitivity was also compromised in *mARF16*/*abi3* double mutant suggesting that mutual action of auxin response factor 16 and ABA transcription factor ABI3 also play role in dormancy induction (51). Also, it has been reported that auxin induced high accumulation of *ABI5* protein during seed germination acted downstream of *ABI3* to inhibit the seed germination which indicate that auxin enhancement of seed dormancy and *ABI3*-dependent ABA seed germination inhibition (132). Hussain et al (88) reported that auxin signaling repressor; IAA8 promoted seed dormancy release in *Arabidopsis* by down-regulating of *ABI3* transcription, thereby further establishing that auxin signaling regulates *ABI3* transcription and that auxin signaling/*ABI3* synergistically inhibit seed germination during dormancy period. The ultimate determinant of dormant status of any part of plant that has potential to germinated is the GA/ABA ration. It has been reported that exogenous auxin treatment repressed soybean seed germination by enhancing ABA biosynthesis, while impairing GA biogenesis, and consequently reduced GA_1/ABA and GA_4/ABA ratios ((122). Consistent with this, ABA biosynthesis inhibitor fluridone reversed the dormancy-induction phenotype associated with auxin treatment, while placlobutrazol a GA biosynthesis inhibitor, inhibited seed germination phenotype due to its action on GA biosynthestic pathway(51). Further quantification of GA and ABA under exogenous auxin treatment, showed that auxin significantly increased ABA content, whereas, bioactive GA_1 and GA_4 levels were decreased, resulting in significant reduction in GA_1/ABA and GA_4/ABA ratios (153). These studies have shown that auxin is exert its influence on dormancy induction and maintenance in plant by mediating ABA and GA biosynthesis and consequently determining the GA/ABA ratio in plant at any point.

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Table 1: Major genes involved in dormancy regulation in crops, their effect on dormancy and action pathways

Gene Name	Effect on dormancy	Action Pathways/Signaling Network	Reference
ZEP	Induce	*Regulates the first step of ABA biosynthesis*	(89)
NCEDs	Induce	*Regulate conversion of all-trans-violaxanthin to 9-cis-voilaxanthin or 9-cis-neoxanthin during biosythesis*	(42)
ABIs	Induce	*Regulate conversion of xanthoxin to abscisic acid aldehyde*	(88)
AAO	Induce	*Mediate conversion of abscisic acid aldehyde to abscisic acid (ABA)*	(91)
AREB; PYR/PYL/RCAR; SnRK2; PP2C	Induce	*Mediate the core ABA signaling networks*	(96-98)
GGDP; CPS; KAO, KO; KSI	Break	*Regulate different stages of GA biosynthesis*	(105, 111, 116)
2ODDS; GA20ox	Break	*Mediate GA1/GA4 transformation to GA2o/GA9*	(119)
ARF10; ARF16	Induce	*Upregulate ABI3 transcription*	(122)
PIF4	Induce	*Regulate the crosstalk between environmental signals and auxin signaling*	(137)
RGL2/SPY	Induce	*Repress GA activity by stimulating ABA biosynthesis*	(125)
DOG1	Induce	*Mediate the crosstalk between ABA-GA by upregulating ABI5 transcription and repress GA biosynthesis*	(138)
SPATULA	Induce	*Inhibition of GA biosynthesis*	(139)
MFT	Induce	*Mediate the crosstalk between ABA and BR biosynthesis pathways*	(140, 141)
BIN2	Break	*Negative regulation of BR signaling network*	(142)
TaBSK2	Break	*Upregulate BR signaling networks*	(143)
TaDET2, TaDWF4	Break	*Upregulate Brassinosteroids (BR) biosynthesis*	(144)
SINL1, SINL2	Induce	*Regulate the expression of Histone proteins transcription factors*	(92, 145)
ACO	Break	*Ethylene biosynthesis*	(146)
ETR1, EIN2	Break	*Ethylene biosynthesis*	(147, 148)
WRKY41	Induce	*Upregulation of ABI3 transcription*	(104)
MYB96	Induce	*Positive regulation of ABI4, NCED2 and NCED6 transcription*	(92)
CYP707As	Break	*Gibberellins' biosynthesis and response to environmental signals (light and photoperiod during dormancy breaking*	(115)
KYP/SUVH4	Break	*Repression of ABI3 transcription*	(53)
LDL1,2	Break	*Downregulation of ABI2, ABI3 and ABI5 transcription*	(149)
YUC	Induce	*Auxin biosynthesis*	(41)
SnRK1	Induce	*Sugar, auxins and ABA regulatory network*	(150)
C/S1 bZIP	Induce	*Low sugar responsive pathways*	(151)
CYCD3	Break	*Regulate cell cycle*	(105, 152)

In addition, IAA has been shown to be a target of two different histone acetyl transferases, specifically auxin influx carrier *LIKE AUXI RESISTANT2* (*LAX2*) and general control nonderepressible 5 (GCN5), which indicates that the *Aux/IAA* genes can also be regulated by epigenetic modifications, and epigenetic modifications also play important role in regulating the expression levels of *Aux/IAA* genes (154), for instance, the transcription factor; *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) can promote the expression of *IAA19* and *IAA29* by directly binding to their promoters to repress the activity of ARF, thereby negatively regulating

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phototropism and auxin signaling (137). Studies have revealed that 21 of 29 *Aux/IAA* genes are the targets of the three *PIFs* (*PIF3, PIF4, PIF5*), and 12 *Aux/IAA* genes are upregulated in response to natural shade and light (155) Fig 4b). These highlight the crucial roles of *Aux/IAA* genes in auxin-mediated light, photoperiod responses; two environmental signals that greatly influence dormancy induction and duration in crops, especially in tubers. Altogether, it has been demonstrated that auxin is an emerging master key player in dormancy induction, maintenance and seed germination mechanisms in plant, and that its effect is exerted through crosstalk between it, ABA, GA, their biosynthetic pathways and signaling networks, as well as environmental signals (light and photoperiod). This plasticity of means of auxin action will also provide opportunity for effective manipulation of undesirable long dormant phenotype of crop like yam, through genetic engineering by targeting any of the phytohormone biosynthetic pathways or signaling networks regulated by auxin which might not be detrimental to tuber yield and food quality. The table 1 above shows some key genes involve dormancy regulation, the nature of their effect on dormancy and their action pathways that have been reported in many crops. Many of these genes and action pathways have been utilized in genetic engineering the crops of interest to modify their dormancy duration.

d) Roles of sugar metabolism in dormancy regulation

As autotrophic organisms, plants produce sugars in mature photosynthetic parts (source organs) to support storage and growth in sink tissues. These sugars drive growth by serving both as metabolic substrates and as signals that tightly interact with hormonal, environmental, and other metabolic cues to coordinate cell growth in specific tissues with storage and nutrient remobilization (64). In doing so, sugars have been linked to stress responses and growth control mechanisms, and an increasing number of studies also implicate sugar signals in developmental decisions such as dormancy induction, senescence, germination and flowering (156-158). The primary sugars in plants are sucrose, glucose and fructose, while sucrose is the primary product of photosynthesis, glucose and fructose are products of breakdown of sucrose by trehalose-6-phosphate (T6P) (150, 151). However, glucose and sucrose are the main metabolic sugars that are widely distributed in plants, and have been recognized as pivotal in integrating regulatory molecules that control gene expression related to plant metabolism, stress responses, and growth and development relented processes including seed dormancy, germination, floral transition, fruit ripening, embryogenesis and senescence (43, 151, 159, 160).

Over the years appreciable progress has been made towards understanding and identifying the dominant plant growth regulatory systems that are influenced mostly by sugars and sugar derived metabolic signals. The sugar signaling pathways in plants can be divided into two groups; (1) those that promote growth and are responsive to optimum sugar availability, include; the hexokinase *(HXK*) glucose sensor, the trehalose-6-phosphate (*T6P*) signal, and target rapamycin (TOR) kinase; (2) those that inhibit growth and are responsive to sugar starvation (deficiency) condition include; sucrose non-fermenting 1 related protein kinase (*SnRK1*) and *C/S1 bZIP* transcription factors (48, 49, 150, 151, 161, 162). The induction of the later pathway is a response to energy deficient (sugar starvation) situation which results in growth arrest. It can be speculated that the same sugar (sucrose) starvation condition is responsible for tuber dormancy induction at the onset of vine senescence of yam crop, during which sucrose photosynthate translocation from the source (leaves and stem) to sink (tuber) is stopped as result of senescence, and to maintain life of the tuber without continuous photosynthate sucrose supply, the tuber might resort to activation and adoption of low energy pathways to ensure optimum utilization of the available sugar by maintaining minimum biological activities associated with tuber dormancy. This argument is supported by the fact that two genes (*SnRK1; C/S1 bZIP*) which are implicated to be positive regulators of the low energy (sugar starving) responsive pathway, have also been implicated to be positive regulators of seed dormancy induction through ABA and auxin/IAA regulatory networks respectively (42, 98, 150, 151, 163).

e) Crosstalk between sugar signaling and phytohormone signaling networks in dormancy regulation

There have been reports about the existing crosstalk between the sugar growth promoting and inhibiting pathways and phytohormone signaling networks which systematically coordinate the molecular; biochemical; physiological and genetical plant growth and developmental processes. For instance, it has been reported that physiological relevant concentration (between 1μM and 10 μM) of T6p (the universal signal of sucrose in plants) inhibits *SnRK1* transcription, and therefore any small changes in T6P concentration within the physiological relevant range produces large changes in *SnRK1* activity, resulting in metabolic reprogramming of hundreds of genes involved in regulation of growth and defense responses (164-167). In the absence of *T6P, SnRK1* regulates the expression of genes that regulate catabolic processes which are important for growth inhibition in a sucrose deficient condition to prevent acute starvation and death. Similarly, glucose-1-phosphate (G1P) and glucose-6-phosphate (G6P) also inhibit *SnRK1* transcription at concentration levels (480 μM, and > 1mM) respectively,

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however, it is only optimum physiological relevant concentration of sucrose that maintain strong inhibition of *SnK1* activity (168, 169). Furthermore, *SnRK1* negatively interact with another sugar growth promoting signaling pathway regulator (target of rapamycin) *TOR* in regulation of plant sugar and growth. SnRK1 downregulates the activities of *TOR* by phosphorylation of key enzymes involved in nitrogen and carbon metabolism through *bZIP* transcription factors, thereby decrease *TOR* activity that causes accumulation of sugars and amino acids (170-172). Thereby revealing the integration of activities of sugar sufficient growth promoting signaling pathway (*T6P; TOR*) and sugar deficient growth inhibition signaling pathway (*SnRK1*) plant growth and development regulation. Figure 6 below shows the two glucose pathways and their crosstalk with plant phytohormones in dormancy regulation

SnRK1 is also a key player in crosstalk between sugar signaling pathways and hormonal regulatory networks in dormancy induction and regulation. For example, in Pea, postembryonic silencing of SnRk1α through a seed storage protein promoter result in defective cotyledon development and seed maturation, including reduced accumulation of protein reserves, impaired desiccation tolerance and viviparity (173, 174). These effects have been reported to be accompanied by altered expression of genes related to cell proliferation and differentiation, leaf polarity and seed maturation, such as *FUSCA3* and *ABI3* (170). Also, *SnRK1* repression reduces the accumulation of cytokinin and ABA (173), thereby impacting on the auxin/cytokinin ratio, another critical factor in plants' decision on root and shoot growth and revealing a link between sugar signaling pathways and hormonal regulatory networks in dormancy induction. In *in vitro* study, *SnRK1* phosphorylates *FUSCA3* transcription factor, but *FUSCA3* degradation was delayed in cell extracted from *35S:SnRK1α1* mutant plants. Furthermore, *35S:SnRK1α1 fusca3-3* double mutant plants display precocious germination and desiccation intolerance similar to *fusca3-3* single mutant plants (158, 170), indicating that *SnRK1* induce dormancy by stabilizing *FUSCA3*. Sugar signaling-ABA induced growth arrest phenotype in Arabidopsis has been screened on high sugar containing medium (6% glucose). This has led to elegant characterization of mutants that are insensitive to sugars. Surprisingly, many of these mutants have defects in ABA biosynthesis or signaling (175), in fact the allelic mutants were identified to be ABA synthesis (aba) and ABA insensitive (abi) in Arabidopsis (176). The role of ABA in plant glucose signaling was described by the characterization of glucose insensitive 5 (gin5) and glucose insensitive 6 (*gin6*)/sucrose uncoupling 6 (*sun6*)/ sugar insensitive 5 (*sin5*) as mutant alleles of aba3 and abi4 respectively (177, 178). In addition, while ABA insensitive 5 (*abi5*) displayed a glucose insensitive phenotype, over expression of *ABI5* results in hypersensitivity to sugars(179, 180). Also, *ABI5* encodes a transcript factor that belongs to the basic leucine zipper *(b/ZIP*) family, and ABA-responsive element binding factors ABF3 and ABF4, two members of *b/ZIP* domain family are strongly induced by ABA (179, 181), suggesting that the role of *ABI5* in glucose-mediated dormancy induction partially overlaps with those other *b/ZIP* factors(175) (182). Two models can possibly explain the overlap between sugar and ABA signaling. That high sugar levels may trigger enhanced ABA synthesis which in turn activates ABA signaling, or that ABA signaling activates shared targets of a separate sugar signaling pathway. This synergistic interaction between ABA and sugar signaling is supported by the fact that ABA alone cannot regulate the expression of some sugar-dependent genes, although it has defining enhancing effect when provided with sucrose (183).

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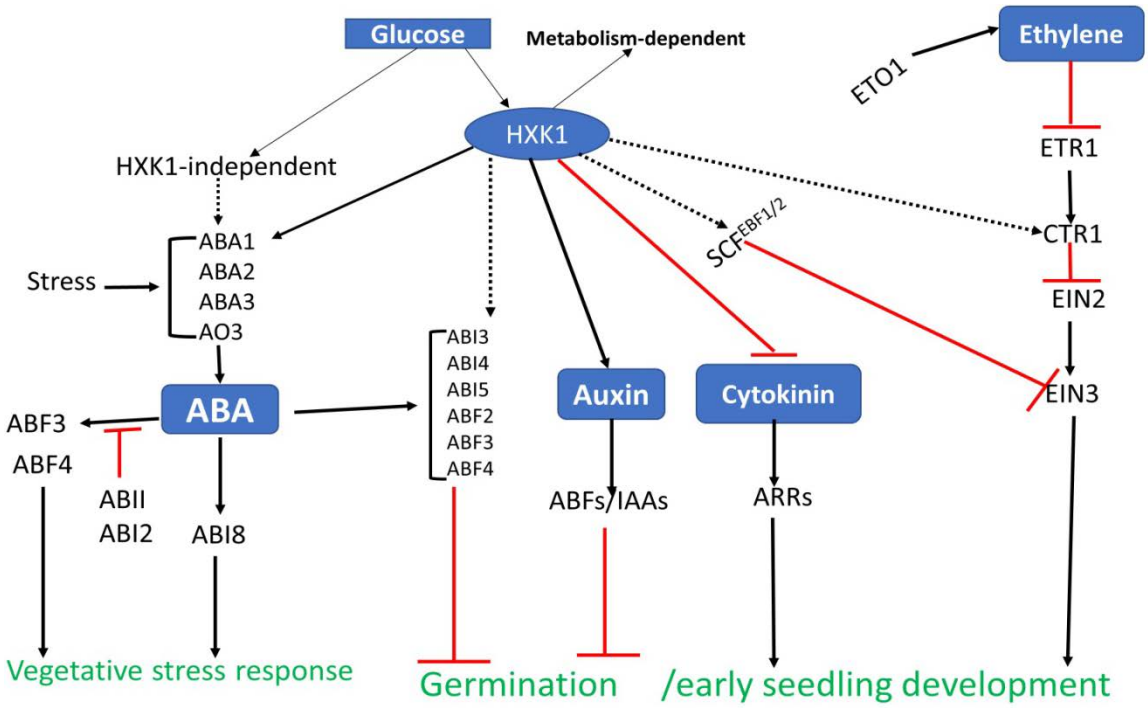

Fig. 6: Hypothetical model of genetic interactions between sugar and hormone signaling. *HXK1*-mediated glucose signaling that regulates dormancy induction, germination and seedling development by inducing both ABA biosynthesis and ABA signaling gene expression. Glucose and ethylene signaling converge on the *ETHYLENE INSENSITIVE3* (*EIN3)* TF to differentially regulate its protein stability. *HXK*-signaling interacts positively and negatively with auxin and cytokinin signaling respectively. Hypothetical connections are shown with *dashed lines*, while connections that led to biosynthetic or regulatory product and developmental trait are shown with arrows, whereas connections that result in repression of either biosynthetic or regulatory product and developmental trait are shown with arrow bars. *Source: Smekeens, et al, 2010*

The multi-level interactions between auxins, cytokinins and sugars are highly complex and are yet to be well understood, even in Arabidopsis. However, some studies have tried to link sucrose to the auxin biosynthesis (184-186), a strong candidate for a long-distance signal promoting lateral root growth. It has been suggested that auxin biosynthesis is induced by soluble sugars, this is support by the fact that daily fluctuations in sugar content highly correlated with fluctuations in auxin levels (184), and circadian clock is responsive to auxin treatment (187). Glucose treatment of Arabidopsis seedlings induces expression of multiple genes encoding auxin biosynthetic enzymes, including; *YUCCA8* and *YUCCA9* (184), corroborating another report that a putative maize *YUCCA* gene is strongly induced by glucose (186). Surprisingly, sucrose effects on auxin levels are more pronounced in the roots than in shoots, suggesting that sugars may impact auxin transport and pathways as well. The growth promoting effect of sucrose is likely through its effect on auxin, as it can be partially mimicked by directly adding auxin and can be blocked by adding polar auxin inhibitors (185). Auxin signaling has also been linked to sugar metabolism. For instance, down -regulation of tomato auxin response repressor *SIAF4* led to a dramatic increase in chloroplast number and an increase in sugar and starch content in the fruit (188). Sugars and cytokinin interact during plant growth and development, and these interactions can be both direct and indirect, and involve cell-specific and long-distance interactions (175). Transcript profiling of Arabidopsis seedlings after glucose and cytokinin treatment showed that many genes involved in stress responses and developmental pathways were affected (189). It has been reported that glucose and cytokinin acted both agonistically and antagonistically on gene expression, and glucose had a strong effect on genes involved in cytokinin metabolism and signaling (190). Cytokinin deficiency caused by constitutive overexpression of cytokinin oxidase (*CKX*) gene, leads to drastic changes in root and shoot growth (191), though molecular mechanisms are only partly known, and involve changes in the cell cycle and in photosynthetic activity, altered carbohydrate distribution and source/sink relations.

Gibberellins (GA) daily fluctuations is also responsive to fluctuations in sugar levels and are regulated by Circadian clock (187, 192). Studies have shown evidence that sucrose stabilizes growth repressor protein (*DELLA*) exert its repression effects by blocking GA regulatory networks (*PIFs*) from interacting

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with environmental signals (temperature and light) which are required to stimulate GA biosynthesis (Fig4b). This provides an explanation for the negative effects GA on the sucrose-dependent induction of the anthocyanin biosynthetic pathway (193, 194). Loreti et al (195) showed that GA repress the expression of several sucrose-induced genes involved in the anthocyanin synthesis (195). Conversely, the repressive effect was drastically reduced in gai mutant expressing a stabilized DELLA protein, indicating that *DELLA*s are involved in the Suc-GA interaction (195). Li et al, (2014) showed that sucrose, not glucose, stabilizes the *DELLA* protein repressor of GA (*RGA)*, given that *DELLA* proteins are stabilized by sucrose and sucrose content increased in plant during the day due to photosynthesis, it will be tempting to speculate that increased *DELLA* level during the day is positively correlated with increased sucrose level during the day (192). But contrary to this, a high growth rate during the day was observed in a starchless mutant that displays high sucrose levels during the light period (196). This increase in growth during the day when sucrose content is rather high contradict the growth repressive effect expected from the sucrose-GA interaction and suggests that there could be other pathway(s) than GA pathway which sucrose is not responsive that drive the high growth rate observed.

Gene set Enrichment Analysis (GSEA) in Arabidopsis, poplar and grapevine dormant buds revealed a very significant enrichment of genes responsive to *AKIN10*, one of the catalytic subunits of *SnRK1*, among them, were robust bud dormant markers such as histone HISI-3 and *DORMANCY1* (197). Also, the *SnRK1* regulatory subunit *AKINBETAI*, whose mRNA levels correlated directly with dark period duration induced buds dormancy (198). *SnRK1* activates autophagy, controls senescence, down regulates anabolism, cell division and protein synthesis (52, 197-199), which are all parameters that characterize dormant buds and were as expected observed in buds entering dormancy. Theses observation further highlight the potency of sugar-SnRK1 interaction mediated dormancy induction.

f) Cell cycle and dormancy regulation

Eukaryotic cell cycle consists of mainly five phases (G0, G1, S, G2, M), each phase shares a set of unique activities in the division of labour that cumulate in cell reproduction. Mitogenic signals are required for completion of cell cycle in each phase, but most especially during the transitions from G1 to S (DNA synthesis) phase and G2 to M phase; for proper coordination of activities and precise progression of the cycle (145), otherwise the cell cycle will experience defects which often lead to different biological phenomenon such as; different degree of ploidy. Different plant hormones and sugars act in crosstalk during cell cycle to induce dormancy by causing cell arrest in G1 phase and subsequent release during germination. Earlier studies have shown that in plant meristematic cells, sucrose deficiency induces endogenous principal control points (PCPI and PCP2), which block cell cycle at G1 and G2 respectively (200-203), this cycle blockade or arrest is what that constitute dormancy induction and is reversible during germination. It has been shown to be reversed by sucrose application which switch on the cell cycle process again though with a delay. The molecular mechanisms regulating the action of PCP1 and PCP2 in this blockade have not yet been elucidated. However, as stated earlier in crosstalk between sugar signaling pathways, it can be speculated that in yam tuber during senescence which is characterized by sucrose deficiency as result of cut in sucrose supply from non-photosynthesizing senescing vine, low energy sugar signaling pathways (*SnRK1*) and *C/S1 bZIP*) which function in crosstalk with auxin biosynthetic pathway to induce growth arrest in response to low energy condition in plants might have elicited the action of PCP1 and PCP2 to effect the cell arrest. This however need to be properly investigated through an organized study. During this period of temporary growth arrest, it has been reported that numerous phosphorylation and dephosphorylation processes occur, both in metabolic pathways and in regulation of the cell cycle. For instance, at the beginning of regeneration, in the presence of sucrose, meristematic cells are strongly sensitive to inhibitors of protein kinases [Cylin-dependent Kinases (*CDK*)] and protein phosphatases 1 or 2A (*PP1/PP2A*), which further results in prolonged blockade of cell cycle (dormancy) (200, 201, 204). It has been demonstrated that this sensitivity decreases with time, and consequently allow the cells to resume regenerative activities through the action of [Cylin-dependent Kinases (*CDK*)], however, the mechanism that regulate the decrease in sensitivity and reduction in the effects of PCP1, PCP2 and possibly *SnRK1* and *C/S1 bZIP* on the blockade in order to allow the action of Cylin-dependent Kinases (*CDK*) pull through is not yet understood and is vital missing link that will be pivotal in dormancy manipulation through genetic engineering.

During G1 phase, auxin was reported to induce expression of cylin D gene; *cyD3-1* and cylin-dependent kinase gene *CDKA-1,* and to play important roles in *CDKA/CYCD* complex assembling (37). Meanwhile, *KRP1* and *KRP2* transcripts, encoding two of the *CDK* inhibitors were reported to be down-regulated after auxin treatment (152, 205-207), thereby sustaining the phosphorylated *CDKA/CYCD* complex. It is this activation of *CDKA/CYCD* complex that is believed to stimulate the phosphorylation of the transcriptional repressor retinoblastoma-related (*RBR*) protein, and release its target; Adenovirus E2 promoter-binding factor A/B (E2FA/B) and dimerization partner A (*DPA*)

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complex. Through this post-transcriptional regulation, auxin stabilizes the *E2FA/B* and *DPA* complex, which up-regulates the expression of genes essential for initiating the S phase (208), and thereby initiating the process of dormancy release. Hence, the growth inhibition in the dormant tuber meristem is a consequence of the arrest of tuber meristem cells at the G1 phase of the cell cycle. Cytokinin (CK) also play role in dormancy regulation at cellular level. It has been demonstrated that exogenous application of CK stimulates tuber dormancy breaking (115, 209), and endogenous CK can initiate the onset of dormancy release. Studies have revealed that exogenous application of zeatin upregulate *CYCD3* in Arabidopsis and Camellia (115, 152), suggesting possible crosstalk between cytokinin, auxin and sucrose in activation of cyclin D genes during dormancy release. During the transition from dormancy to dormancy breaking phase of tuber, expression of genes encoding histone proteins (H3, H4, H2B) and other proteins such as *MAP* kinase, γ tubulin, and ovule/fibre elongation protein have been implicated in cell division and initiation of dormancy breaking (206). The implication of histone proteins (H3, H4, H2B) during cell division process is quite expected, because these histone proteins are the DNA packing materials and during synthesis or replication, the DNAs are unpacked thereby releasing the packing materials (histone proteins). Furthermore, histones also function as receptors of environmental signals (temperature and light) which act through phytochromes signaling (PIFs) to induce gibberellins (GAs) biosynthesis (Fig4), which in turn initiate dormancy release and germination process.

IV. Conclusions and Future Perspectives

Dormancy and sprouting are important stages of tuber development providing for successive vegetative growth and regeneration of yam tubers. Characteristics of tuber dormancy, its duration in particular, are stable hereditary traits. Tuber dormancy and sprouting include a complex of different, but coupled physiological and biochemical processes. The main ones are growth and its active blocking, as well as storage and active usage of sugars and proteins. Though, how these processes are integrated at the molecular, physiological and genetic levels and how they are coordinated with each other in regulation of dormancy induction and germination have been extensively studied using modern tools in other crops including potato tuber and the processes are highly conserved across crop species, but in yam crop such studies are still lacking. Such studies are particularly important in yam crop, in view of long dormancy duration phenotype of its tuber, which has constituted a major constraint in yam research and genetic improvement and consequently imped unlocking of its productive and utilization potentials. It has been established through elegant studies that the process of dormancy induction and breaking is a complex, separate, but continuous physiological and molecular processes involving wide range of hormones, sugars, cellular activities and their regulatory networks crosstalk, leading to expression of many genes that function in a coordinated manner to determine crop phenotype with regards to dormancy duration and germination. It was demonstrated that Abscisic acid (ABA), Gibberellins (GAs), Auxins, sugar signaling pathways and their regulatory networks crosstalk are the key master players in regulation of crop dormancy and germination. Particularly, it has been shown that sugars, non-fermenting related kinase 1 (*SnRK1*) and to lesser extent basic leucine zipper (b/ZIP) group of protein motifs play prominent roles in all the major dormancy induction and maintenance regulatory pathways, for example, in ABA, GA, Auxins, Low sugar signals and cell cycle active blocking at G1 phase, *SnRK1* and b/ZIP are involved and their actions are also conserved across plant species so far studied. Therefore, focusing on their roles in search of solution to long duration dormancy phenotype of yam tuber, might provide veritable opportunity for tuber dormancy to be manipulated to fit the agronomically desired tuber dormancy phenotype, through genetic engineering of any of the regulatory networks without yield and food quality trade off.

Declarations

Ethical Approval

Not applicable

Competing Interest

The authors have declared that there's no competing interest associated with this article, in any financial, patent ownership and personal relationships

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Physical and Mechanical Properties of Teak (Tectona Grandis L.Fil.) Thermo-Plywood from Plantations in Lao P.D.R

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Abstract- The aim of this research to investigated physical and mechanical properties of thermal plywood of PF adhesive. Physical property focused on density, water absorption and swelling. And mechanical property was MOR, MOE, shearing strength and hardness. The Teak veneer was the main raw material the size was 2 mm in thickness, and 400 mm in width and length. The veneer was divided into five groups as were control, thermos-veneer, at 180°C, at 200°C, 220°C and 240°C and 4 min, 8 min and 12 min, the spread 150 g/m2 and five layers. Plywood products used PF adhesive products were cool pressing in 10 min and hot pressing in 15 min. density test followed with AS/ANZ 2098.7, 2012, WA and swelling thickness were followed ASTM D 3502-76, The dimension sample design was 10x25x300 mm for MOR and MOE, 10x50x100 mm for shearing strength and 10x50x50 for harness and testing by Fast Test software. Data analyses were One-way ANOVA and multiple linear regression. *As the result, the density of* thermal treatment was decreased density of teak plywood. A control was significant different on thermal treatment of different level of temperature and length of time the control was no significant different.

Keywords: thermo, plywood, density, absorption, swelling, MOR, MOE, shearing, hardness.

GJSFR-G Classification: DDC Code: 634.97396 LCC Code: SD397.Z35



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Abstract- The aim of this research to investigated physical and mechanical properties of thermal plywood of PF adhesive. Physical property focused on density, water absorption and swelling. And mechanical property was MOR, MOE, shearing strength and hardness. The Teak veneer was the main raw material the size was 2 mm in thickness, and 400 mm in width and length. The veneer was divided into five groups as were control, thermos-veneer, at 180°C, at 200°C, 220°C and 240°C and 4 min, 8 min and 12 min, the spread 150 g/m2 and five layers. Plywood products used PF adhesive products were cool pressing in 10 min and hot pressing in 15 min. density test followed with AS/ANZ 2098.7, 2012, WA and swelling thickness were followed ASTM D 3502-76, The dimension sample design was 10x25x300 mm for MOR and MOE, 10x50x100 mm for shearing strength and 10x50x50 for harness and testing by Fast Test software. Data analyses were One-way ANOVA and multiple linear regression. *As the result, the density of* thermal treatment was decreased density of teak plywood. A control was significant different on thermal treatment of different level of temperature and length of time the control was no significant different. WA in the different level of temperature and length of time the WA increases. The control was significant different with thermally. Swelling thickness of thermal treatment at 180, 220, 240 °C was increased to significant different with control, and length of time the control was no significant different. The MOR in different level of temperature the control was only significant with at 180°C and but another group at 200, 220, and 240°C was no significant. The length of time on 4, 8, and 12 min was no significant different. MOE was decreased in different level of temperature at 180, 200, 220, and 240°C significant different and length of time on 4, 8, and 12 min was no significant different. The different level of temperature effected MOE of teak plywood. The shearing strength in different level of temperature at 180, 200, 220, and 240°C and length of time on 4, 8, and 12 min were no significant different. And the hardness in thermal treatment at 180, 200, 220, and 240°C and length of time on 4, 8, and 12 min was fluctuated. And it was no significant different.

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Keywords: thermo, plywood, density, absorption, swelling, MOR, MOE, shearing, hardness.

I. Introduction

Teak plantation quality is most important for sawmilling where processors acquired high volume from the processing. Lao teak plantations were abandoned naturally but Luang Prabang Teak Program (LPTP) provided technical skills in maintenance like thinning and pruning for local farmers (Fondation Ensemble, 2014).,(Bouaphavong *et al.*, 2016) confirmed that planted teak in Laos had many knots which affected log grad compared to grown teak in Thailand. GPérez and Kanninen (2005) confirmed that Teak wood quality is of considerable importance when classified according to international grading rules. Since teak is a light-demanding tree light-demanding does grow not grown well in dense stands (Budiadi. *et al.*, 2017).

DJATI *et al.* (2015) also has done research on mechanical property and characteristic of young teak for making products in Indonesia. The teak wood contains different proportion of sapwood and heartwood depend on ages, such as young teak wood has lots of sapwood but rich of heartwood in old trees. Proportion of heartwood teak was 91% during 50 to 70 years old in the East Timor (Miranda *et al.*, 2011). Izekor *et al.* (2010)confirmed that teakwood density and mechanical properties increased with increase in ages. The research comparison between heartwood and sapwood in density and MOR, which density of teak ages for 1-10 years old the sapwood and heartwood were 0.61 and 0.63 g/cm^3, 11-20 years old were 0.65 and 0.61 g/cm^3, 21-30 years old were 0.62 and 0.72 g/cm^3, and 31-40 years old the size of sapwood less than minimum standard and heartwood was 0.75 g/cm^3. For MOR testing between sapwood and heartwood in 1-10 years old were 52.70 and 80.26 MPa, 21-30 years old were 91.41 and 81.45 MPa, and 31-40 years old the sapwood less than standard and heartwood was 101.10 MPa. The conclusion teak ages to most important with properties of teak.

According to Sedliacik *et al.* (2010) studied that compared low temperature pressing of 100 °C in 6 min,

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glue spread of 140 g/m^2 with plywood pressed by high temperature (120 and 150°C) by phenol formaldehyde condensation. The results showed that lower temperature pressing was significant without worsening of mechanical properties of plywood. Moisture content of veneer was 10-12%, 2 mm thick, it used cornstarch-tannin-phenol formaldehyde amount 225 g/m^2, at 125°C in 6 min, loading presser was 12 bars as the result of MOR was 41 N/mm^2 of the control, 70 N/mm^2 of the cornstarch PF, MOE was 2,958 N/mm^2 of the control, 4,271 N/mm^2 of the cornstarch PF, strength tension in 8 hours boiling water was 0.5 N/mm^2 of the control, 1.75 N/mm^2 of the cornstarch PF, wood failure was 11% of the control, 72% of the cornstarch PF and formaldehyde emission was 2.62 mg/m^2/h of the control, 1.92 mg/m^2 /h (Moubarik *et al.*, 2009). Lignin phenol formaldehyde resolves were prepared different of lignin at both 20 and 40% phenol replacement (Ghorbani *et al.*, 2016). Phenol formaldehyde was low formaldehydes effectives with products and user, high water resistance, it was developed for outdoor plywood and PF was used improve teak plywood in Laos and supporting information to Lao industry.

Thermo wood modification method has been found since early part of the 20th century (Callum, 2011). Wood modification could be the best option for improving teak plantation in Laos. For instance, thermo-plywood can increase durability and dimensional properties of the product and it can also reduce chemical use for wood preservation. For example, worldwide is concerning environmental effect, especially, European zone is high restricted use of toxic preservation and the market for new durable products of modified wood during the last few years(Sandberg *et al.*, 2017). Even though thermal modification is long-known method, considerable amount of research has recently focused on this method and heat-modified wood has an important market share (Kantay & KARTAL, 2007). Thermo-wood modification can be performed in special thermal treatment kilns as well as under pressure and heat using special press equipment. Considering special thermal treatment kilns, five processes have been developed and are currently available at industrial scale. Thermo-plywood products can extend young teak duration, safe for users and environmentally friendly because thermo-veneer to be able chemical composition of teak veneer, thermo-plywood never use chemical elements (toxic) which only using glue ability and the product high resistances with insect destroying such termites, beetles, and others.

Generally, Teak log thinned from plantation is less utilization in Laos, which has small diameter and more proportion of sapwood. Famers have been using it for fence, poles, firewood, discard on the ground plantation site and some burning. Young lumber teak is low insect resistance especially termites, fungus, and beetles. The modern alternative to improve wood products needs to be modernized wood technology into wood industrial in Laos. Thermo-plywood method is believed to be able to increase smart teak products without chemical and high insect resistance through removing chemical composition of the wood, thermo-wood is thermal modification. According to Mohamed *et al.* (2018) found that thermo wood, whenincreasing temperature in the range of 160-220 °C. Thermo-Wood® method for about 1 and 2 hours at temperatures of 190 and 212 °C in an industrial business (Aytin *et al.*, 2015). Both research article confirms that thermal domification to be change chemical composition of the wood by temperature increasing rang in the range more 160°C.

This study objectives to investigated physical and mechanical properties of thermal plywood of PF adhesive. Physical property focused on density, water absorption and swelling. And mechanical property was MOR, MOE, shearing strength and hardness.

II. Material

Teak veneer was peeled insplindleless rotary at the Faculty of Forest Science, National University of Laos, Vientiane Capital, Lao.P.DR. A small wood hot pressure machiner (model STK No. 44-275, DAKE, Grand Haven, MI, USA), A Cole pressure machiner (model No BY 814*4/2B, Production code 7265, Made in China), a hot pressure machiner (model, No BY 214*4/2A-1, Production code 7276 Mad in China), a circular saw cutter (model, No BJ 6116-4B, Production code 180401), dial thickness gaugedigital calipers (code 34-506, Measumax, Melbourne, Australia) were used. And Horizontal Flow Oven (Model WOF-050, serial No WOF050071018002, Made by Daihan Scientific Co., Ltd).

III. Methodology

The veneer was cut into pieces measuring 2 mm x 400 mm x 400 mm with a total of 60 pieces of peeled veneer; the moisture content of veneer ranged from 6 to 12% based on air-dry weight. The thermal treatment processes were based on the factorial design as 5 x 13 x 30 = 1,950 (four different temperatures, *i.e.*, 180, 200, 220, and 240 °C; three different times, *i.e.*, 4, 8, and 12 min for the treatment of five peeling veneer at a time).

The adhesive to apply on teak veneer for teak plywood products is phenol formaldehyde based on Standard (2012). Plywood product consists of 5 layers or 10 mm of thickness, veneer laying should be placed over to another with perpendicular grain. PF glue should be speeded on the veneer by passing them through the roller of a glue transfer spreader between 150 g/m^2.

Dimension of teak plywood product is 40x40 cm which it is hot press machinery. The hot press rate used as 1mm thick per 1 min, thermo-teak plywood and teak

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plywood has 10 mm of average, time for pressing at 10 min for 130 °C. The press loading is 2.5 MPa/mm^2.

A. Density testing: According to the Standard (AS/ANZ 2098.7, 2012), the specimen should be cut into 10x25x50 mm (30 specimens), formula calculation as Eq1.

$$\text{Density } \frac{\text{Mass}}{\text{Volume}} \quad (1)$$

Where: Density is mass divided by volume of a specimen (g/cm^3), Mass is the weight of a specimen (g), Volume is the multiple of thickness, width, and the length of a specimen (cm^3).

B. Water Resistant or Absorption Testing: method for testing was using ASTM D 3502-76 as the method recorded by Somwang (1995). Method testing specimen was placed in a conditioning chamber for 24 h in the temperature at 50±2°C. Next, Specimen should be soak in the distilled water at 25±2 °C in for 24 hours, then using dry cloth to support for taking of water from the surface and immediately weigh it and recording data. Water absorption (WA) calculation as Eq 2.

$$\text{WA} = \frac{W_2 - W_1}{W_1} \text{x} 100 \quad (2)$$

Where: WA water absorption (%), W_1 Weight of a specimen before soaking distilled water (g), W2 weight of a specimen after soaking distilled water (g)

C. Thickness swelling: the method was following up point B mention above, thickness was measuring by digital caliper and swelling changes as calculated in Eq 3.

$$\text{Swelling (\%)} = \frac{(T_2 - T_1)}{T_1} \text{x} 100 \quad (3)$$

Where: T_1 thickness before soaking in distilled water and T_2 Thickness after soaking distilled water.

MOR and MOE were testing the same specimen which it's dimension of 10x20x300 mm accordance with D1037-12 for test specimen shall be less than 12 mm of thickness and speed loading rate is 3mm/min. formula of rate as calculation in the Eq 4.

$$MOR = \frac{3xP_{max}\ xL}{2xbx\ d^2} \quad (Eq4)$$

Where: P_{max}=maximum load (N), L= Length of span (mm), b= width of specimen measured in dry condition (mm), d=thickness (depth) of specimen measured dry condition (mm)

MOE was calculating by formula as Eq 5.

$$MOE = \frac{L^3}{4xbx\ d^3} x \frac{\Delta P}{\Delta Y} \quad (Eq\ 5)$$

Where: L= Length of span (mm), d=thickness (depth) of specimen measured dry condition (mm), b= width of specimen measured in dry condition (mm), $\frac{\Delta P}{\Delta Y}$ =slope of the straight-line portion of the load deflection curve (N/mm)

The shearing strength was following D143. The load applied continuously throughout the test at a uniform rate of motion of the movable crosshead of the testing machine of 0.6 mm/min, the calculation as Eq 6.

$$Shearing = \frac{P_{max}}{bd} \quad (6)$$

Where: d=thickness (depth) of specimen measured dry condition (mm), b= width of specimen measured in dry condition (mm), and P_{max}=maximum load (N)

The diameter of head pressor is 10 mm, and load applied continuously throughout the test at a uniform rate of motion of the movable crosshead of testing of 1.3 mm/min (ASTM, 20112). The calculation hardness as Eq 7.

$$Hardness\ (MPa) = \frac{P}{2¶rh} \quad (5)$$

Where: P=loading selected is to be in calculation (N), r=radian of penetrating ball (mm), and h=depth of penetrating in specimen (mm)

Data Analysis: All properties of teak plywood analysis conducted by One-way ANOVA table of statistic in relativity 95 %. Whole study was use multiple comparison. If Significant (sig) differences start from sig equated 0.05 (p=0.05) and high significant differences of three techniques and wood machineries in recovery is 0.00 (p=0.00). The multiple regression model analyzed only mechanical property.

IV. Result and Discussion

Table 1 showed that average and standard deviation of physical and mechanical property of thermo-treatment teak plywood. The length of time was showed in Table 2, the physical property investigated density, thickness swelling and water absorption. The mechanical property investigated MOR, MOE, Shearing, and hardness.

The density was highest (0.705±0.04 g/cm^3) which it was thermo-treatment in different level of temperature for percentage of density decreased in 3.12% at 180°C, 5.39 % at 200 °C, 3.12% at 220 °C, and 3.40 % at 240 °C compared with control CL. This study the age of teak plywood 18 year old to less than May (2003) study teak age was 20 year old in 0.26 g/cm^3.This study to compared with Cuccui *et al.* (2017) treated solid wood at temperature of 180°C for 5 hours, the PF density of this study was higher 12.88% in condition of humidity at 0%, 11.27% in condition of humidity at 30 %, and 9.80 % in condition of humidity at 60%.

One-way ANOVA analysis to compared between significant different from each temperature level, result showed that PF plywood product compared between control with thermal at 180-240°C was high significant different P <0.05. While compared temperature between 180°C with 200-240°C was only

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200°C was high significant different but 220 °C and 240 °C were not significant different. The length of time of PF density was 0.705±0.040 g/cm^3 in CL, 0.676 ±0.041 g/cm^3 in4 min, 0.679±0.038 g/cm^{3}in 8 min, and 0.681 0.±033 g/cm^3 in 12min. One-way ANOVA analysis. the result illustrated that different length of times thermal treatment as the control compared between group (4 min, 8 min and 12 min) were significant different, as P<0.05 (P=0.000, P=0.001 and P=0.002) respectively. To compared 4 min with 8 min and 12 min were no significant and 8 min with 12 min was no significant different.

The water absorption (WA)of plywood showed that it was 31.07±4.60, to compared control with different level of temperature at 180 °C for PF was increased 6.05%,thermo-treatment at 200°C was increased 1 %, thermo-treatment at 220°C was increased 4.9 %, and thermo-treatment at 240°C increased 2.55 %, According to Uribe and Ayala (2015) confirm that WA of treatment solid teak has 25 mm thick for 2 hour in different level of temperature, it was 4 % at 180°C, 3.6 % at 200 °C, and 2 % at 210 °C as much different of this study. In contrast, this study was similar with Islam *et al.* (2012) studied WA in plywood of *Eucalyptus camaldulensis*, the WA was 36.9±3.82 %. DEL MENEZZI *et al.* (2017) studied that the WA of teak plywood absorption were improved depend on volume of gluing speed as 0.96 g/m^2, 1.92 g/m^2, and 2.88 g/m^2, the WA were 36.08 %, 30.26 %, and 25.20 %. Thermo-teak plywood in this study used gluing speed was 150 g/m^2 the WA range from 31.07-37.12 % for PF, 31.49-37.26 %. In conclusion, thermal treatment was not improved WA, but volume of gluing speed was significant for plywood.

One-way ANOVA in Duncan analysis the water absorption which it groups a was significant different with b (a=180°C, 220°C and b=control, 200°C, 240°C). One-way ANOVA analysis the variable multiple comparison of times length different for teak thermal plywood, The between group to compared control with 4 min was high significant different (P=0.00), to compared control with 8 min was significant different (P=0.02), to compared control with 12 min was no significant different (P=0.45), to compared 4 min with 8 min was high significant different (P=0.00), to compared 4 min with 12 min was high significant different (P=0.00) and to compared 8 min with 12 min was significant different (P=0.01).

The swelling thickness of thermo-teak plywood showed that the control was 3.08±1.12 %, the thermo-treatment at 180, 220, 240°C was increased from a control of 1.48,0.46, 0.51 % respectively. But at 200 °C the swelling thickness comparison with control was decreased 1.36 %. Bas on Uribe and Ayala (2015) showed the swelling thickness of teak treatment at 180 °C was0.8 %, at 200 °C was 0.6 %, and 210 °C was 0.5 % which it was decreased in different level of temperature but teak thermo-plywood of this study was not change. The mean value of eucalyptus plywood (1.5%) was evidently lower than that of simul plywood (2.9%) (Islam *et al.*, 2012). This study was higher Ayala (2015), and Islam *et al.* (2012) because the high temperature was changed physical property of thin veneer. However, water has been absorbed as well.

Base on Duncan in One-way ANOVA analysis grouping significant different, the thickness swelling thermal treatment at 200°C was significant different with control, 220°C and 240°C (P=0.00) and 180°C, but control compared with at 220, and 240°C was no significant different (P=0.41, 0.36) respectively. And to compared 180°C with control sample was significant different (P=0.01), at 200°C was significant (P=0.01) and at 240°C was significant different (P=0.01). The length of time illustrated that the control compared with 4, 8, and 12 min was no significant different (P>0.0.05), to compared 4 min 8 min sample was significant different (P≤0.05), but on 4 min compared with 12 min was no significant different (P>0.05), and 8 min with 12 min was high significant different(P<0.05).

Base on this study found that the PF average MOR of control sample was 68.33±20.59 MPa, it was higher than SOUKPHAXAY *et al.* (2021) studied teak LVL in 6.79 MPa. According to Sutiawan *et al.* (2021)studied that the influence of different hot pressing condition of Jabon plywood, the MOR perpendicular parallel to grain was increased at high hot pressing i,e., 34.78 MPa at180 °C, 57.51 at 190 °C, and 58.60 MPa at 200°C. This study discussed at 180 °C, and 200 °C was increased in 8.55 MPa of different. But Sutiawan *et al.* (2021) confirmed that the MOR different between 180 °C to 200 °C was increased 23.82 MPa. The thermo-treatment at different level of temperature was unchanged MOR of the plywood. The MOR of teak LVL used volume of gluing speed in 0.96 g/m^2, 1.92 g/m^2, and 2.88 g/m^2 the result was 49.3 MPa, 53.6 MPa, and 74.1 MPa respectively(DEL MENEZZI *et al.*, 2017). However,The MOR of thermal treatment in different level of temperature were not reduced force loading in the plywood, types and volume spread of adhesives were significant to static bending.

MOR in control sample with 180°C was significantly different (P<0.05), to compared control with 200°C, 220°C, and 240°C samples were no significant differences (P>0.05), to compared 180°C samples with 200°C220°C and 240°C samples were significantly different (P<0.05), to compared 200°C with 220°C was no significant different (P>0.05), to compared 200°C with 240°C was significantly different (P<0.05) and to compared 22°C with 240°C was no significant different (P>0.05). MOR of PF group confirm that heat treatment with high temperature was impacted with MOR of teak plywood. The length of time on control compared with 4, 8, and 12 min the result was not significantly different (P>0.05).

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The modulus of elasticity MOE of teak plywood thermal treatment of control was 12,734.54± 2,246.33 MPa, the thermal treatment was reduced MOE on high different level of temperature as to compared control with at 180 °C, 200°C, 220 °C, and 240 °C the MOE. According to DEL MENEZZI *et al.* (2017) studied that the teak LVL of MOE in different volume of spread was 8,469.8 MPa, 8,872.4 MPa, and 9,132.6 MPa to lower than this study. SOUKPHAXAY *et al.* (2021) studied that the MOE of teak LVL was lowest in 2,609.97 MPa. DEL MENEZZI *et al.* (2017) and SOUKPHAXAY *et al.* (2021) confirm that the MOE of LVL was lower than teak thermal treatment of plywood. The different level of temperature thermal treatment decreased MOE of teak thermal treatment plywood.

Based on multiple comparisons by One-way ANOVA analysis showed that the results of PF groups to compared a control with 180°C, 200°C, 220°C, and 240°C were high significantly different (sig<0.05), to compared 180°C with 200°C was not significantly different (sig>0.05), to compared 180°C with 220°C and 240°C were high significantly different (sig<0.05), to compared 200°C with 220°C and 240°C were high significantly different (sig<0.05), and to compared 220°C with 240°C was not significantly different (sig>0.05). The multiple comparisons of times length thermal on different times of two plywood adhesives. To compared a control sample with 4 min, 8 min and 12 min were high significant different (sig<0.05), and its MOE were high than other times thermal treatment. Comparing 4 min with 8 min and 12 min was not significantly different (sig>0.05) and to compared 8 min with 12 min was not significantly different (sig>0.05).

The shearing strength value of teak plywood thermal treatment of control in PF was13.55±2.97 MPa. This study was similar Nautiyal (2015) studied that shear strength of *Melia composita and Populus deltoides* in 5 ply, to applied PF condensation and result showed that the shear strength in dry condition ranged from 13.82 to 14.7 MPa, and 9.4 to 11.57 MPa in wet condition. According to International Thermowood Association (2003) reported that the higher-temperature treatments (at 230 °C for 4 hours) the strength properties were reduced in radial tests from 1 to 25%. This study was similar with theory of International Thermowood Association (2003). But this study shear strength was higher Bekhta *et al.* (2016) in 8.39 MPa because this study used PF glue spread more 150 g/m^2. The shearing strength was depended on volume of glue spread in layer plywood.

One-way ANOVA analysis showed that the multiple comparison of significant different for PF. The PF group, to compared control sample with at 180 °C, 200 °C, 240 °C were no significant different (P>0.05), to compared control sample with at 220 °C was high significant different (P<0.05), to compared at 180 °C with at 200 °C was no significant different (P>0.05), to compared 180°C with at 220°C, and 240°C were significant different (P<0.05), to compared at 200°C with at 220°C was high significant different (P<0.05), to compared at 200°C with at 240°C was not significant (P> 0.05), and to compared at 220°C with at 240°C was high significant different (P<0.05). The length of time thermal treatment on 4, 8, and 12 min was not significant different.

The hardness of this study was showed in Table 1, and 2 show the average and standard deviation of teak plywood thermal treatment. The control was 22.09±6.35 MPa the percentage of hardness compared with control showed that the hardness increased at 200°C, and 240°C in 11.18 %, and 0.95 (PF) respectively. In contrast, the different level of temperature at 180°C, 220°C the hardness decreased in 14.21 %, 0.22 % (PF) respectively. The length of time illustrated that the thermal treatment on 4, 8, and 12 min was a little fluctuation. According to Lengowski *et al.* (2021)confirm that teak thermal treatment was significant declines of hardness occurred in the longitudinal and tangential section. Pelit *et al.* (2015) confirm that the higher in Eastern beech samples (36.30 N/mm^2) than in Scots pine samples (27.27 N/mm^2), and the hardness was increased as treatment temperature increases. Base on International Thermowood Association (2003) recorded that the thermo-solid wood treatment at 180°C to 240°C the hardness increased as the treatment temperature increases, the Brinell hardness was highly dependent on the density as bout 17.64 %. This study was similar with Lengowski *et al.* (2021) at 180°C, 220°C (PF), and similar with Pelit *et al.* (2015) and International Thermowood Association (2003) at 200°C, and 240°C (PF).

The multiple comparison analysis of the PF group, to compared control with at 180°C and 200°C were significant different (P<0.05), to compared control with at 220°C and 240°C were no significant different (P>0.05). The significant point is that compared at 180°C with at 220°C 220°C and 240°C were high significant different (P<0.05), but to compared at 200 °C with at 220°C, 240°C were no significant different (P>0.05), and to compared at 220 °C with at 240°C was no significant different (P>0.05). The length of time thermal treatment on 4, 8, and 12 min. To compared control with 4 min, 8 min and 12 min were no significant different (P>0.05), but to compared 4 min with 8 min was high significant (P<0.05), to compared 4 min with 12 min was no significant different (P>0.05), and to compared 8 min with 12 min was significant different (P<0.05).

PF multiple regression model (Eq 5X) considered as high (R^2=0.83)

$$MOR(PF)=0+0.3103*T+(-0.1603)*t \quad \text{(Eq 5x)}$$

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Where: MOR= Modulus of rupture (MPa), T= Temperatures requirement thermally (°C), t= Times requirement thermally (min), (PF)=Phenol formaldehyde UF multiple regression model (Eq 5x) considered above average (R^2=0.79)

MOR(UF)=0+0.3253*T+(-1.5801) *t (Eq 5x)

Where: MOR= Modulus of rupture (MPa), T= Temperatures requirement thermally (°C), t= Times requirement thermally (min), (UF)= Urea formaldehyde, PF multiple regression model (Eq 5X) considered as high (R^2=0.85)

MOE(PF)=0+51.2504*T+(-43.7003) *t (Eq 5x)

Where: MOE= Modulus of Elasticity (MPa), T= Temperatures requirement thermally (°C), t= Times requirement thermally (min), (PF)=Phenol formaldehyde UF multiple regression model (Eq 5x) considered above average (R^2=0.88)

MOE(UF)=0+50.5502*T+(-56.3688) *t (Eq 5x)

Where: MOE= Modulus of Elasticity (MPa), T= Temperatures requirement thermally (°C), t= Times requirement thermally (min), (UF)=Urea formaldehyde,

Table 1: Physical and mechanical properties of teak plywood in the thermal treatment of different level of temperature in PF adhesive

Properties	Temperature (°C)				
	CL±Std	180±Std	200±Std	220±Std	240±Std
Density (g/cm^3)	0.705±0.04	0.683±0.03	0.667±0.03	0.683±0.04	0.681±0.04
Water absorption (%)	31.07±4.60	37.12±6.59	32.07±11.44	35.97±5.54	33.62±5.37
Thickness swelling (%)	3.08±1.12	4.56±2.70	1.72±3.34	3.54±2.85	3.59±2.11
MOR (MPa)	63.33±20.59	58.10±22.85	66.65±20.55	64.55±21.02	65.81±18.06
MOE (MPa)	12,734.54±2,246.33	11,383.85±2,265.14	10,945.29±1,634.48	9,673.65± 2,407.91	9,948.38± 2,229.25
Shearing (MPa)	13.55±2.97	12.53±3.20	12.76±3.22	11.39±3.58	13.51±3.46
Hardness (MPa)	22.09±6.35	18.95±5.11	24.56±6.01	22.04±6.15	22.30±6.78

Table 2: Physical and mechanical properties of teak plywood in the thermal treatment of length of time in PF adhesive

Properties	Time (min)			
	CL±Std	4±Std	8±Std	12±Std
Density (g/cm^3)	0.705±0.040	0.676±0.041	0.679±0.038	0.681±0.033
Water absorption (%)	31.07±4.60	37.56±7.16	34.59±7.04	32.25±9.25
Thickness swelling (%)	3.08±1.12	3.29±2.97	4.00±2.45	2.77±3.28
MOR (MPa)	68.33±20.59	62.93±19.52	66.09±21.90	62.32±21.12
MOE (MPa)	12,734.54±2,246.33	10,887.21±2,000.07	10,487.39±2,331.09	10,088.77±2,370.60
Shearing (MPa)	13.55±2.97	12.74±2.74	12.50±3.87	12.40±3.63
Hardness (MPa)	22.09±6.35	21.09±6.00	23.49±6.54	21.79±6.31

V. Conclusion

1. Density of teak plywood thermal treatment in control, at 180, 200, 220, and 240°C in the length of time in control, 4, 8 and 12 min ranged from 0.667-0.705 g/cm^3, thermal treatment decreased density of teak plywood. A control was significant different on thermal treatment of different level of temperature and length of time.
2. Water absorption of teak thermal treatment in control, at 180, 200, 220, and 240°C in the length of time in control, 4, 8 and 12 min range from 31.07-37.12%. The different level of temperature and length of time the WA increases. The control was significant different with thermally.
3. Swelling thickness of plywood thermal treatment in different level of temperature as control, at 180, 200, 220, and 240°C base on length of time 4, 8, and min was range from 1.72-4.56%. The thermal treatment at 180, 220, 240 °C was increased to significant different with control, and length of time the control was no significant different.
4. The MOR of control sample was 68.33±20.59 MPa the thermal treatment plywood was fluctuated. The different level of temperature the control was only significant with at 180°C and but another group at 200, 220, and 240 °C was no significant. The length of time on 4, 8, and 12 min was no significant different.

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5. The MOE of teak plywood, the control was 12,734.54 MPa, it was decreased in different level of temperature at 180, 200, 220, and 240°C significant different and length of time on 4, 8, and 12 min was no significant different. The different level of temperature effected MOE of teak plywood.
6. The shearing strength value of teak plywood in control 13.55 MPa, thermal treatment in different level of temperature at 180, 200, 220, and 240 °C and length of time on 4, 8, and 12 min was decreased. A control and at 200 °C, 220 and 240 °C were significant different, but thermal treatment at 180, 200, and 240°C on 4, 8, and 12 min was no significant different. The shearing strength was a little decreased.
7. The hardness a control average was 22.09 MPa, thermal treatment at 180, 200, 220, and 240°C and length of time on 4, 8, and 12 min was fluctuated. And it was no significant different.

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Auto Inflammation Candidate Genes in Juvenile Idiopathic Arthritis

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Abstract- Juvenile idiopathic arthritis (JIA) is a heterogeneous pathology with uncertain causative factors and prognosis, stemming from an immune system dysfunction with the development of autoimmune reactions. The most distinctive and potentially most severe of these is systemic JIA (sJIA), a disease characterized by sharp rises in temperature and rash. A thorough understanding of the complex of immune regulatory mechanisms along with genetic analysis reveals complex relationships between autoimmune reactions and auto inflammation. Sequencing of 15 auto inflammatory genes was performed in 62 patients with JIA: 26 – oligoarthritis, 20 – polyarthritis, 16 – systemic. Studies have shown that 16 (25.8%) patients with the clinical JIA phenotype had changes in nucleotide sequence in the genes encoding auto inflammatory immune response proteins. NOD2 changes were in 12 (19.3%) of them and 1 change in each of the 4 patients NLRP12 (heterozygote, c.1343G> C (p.Gly448Ala)), MEFV (pathogenic heterozygous, c.2082G> A (p.Met694Ile)), ADA2 (heterozygote, c.145C> T). Arg49Trp)), PSTPIP1 (heterozygote, c.806T> A (p.Ile269A)) in the group of studied children with JIA. The study will allow identifying individual genetic loci of JIA risk, expand understanding of the pathogenesis and spectrum of phenotypic manifestations of the disease, improve diagnosis and prediction of its course, as well as reveal new opportunities for monitoring patients with JIA and their personalized therapy.

Keywords: *children, arthritis, genes, autoinflammatory.*

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Autoinflammation Candidate Genes in Juvenile Idiopathic Arthritis

Mukvich Olena [α] & Matskevych Anna [σ]

Abstract- Juvenile idiopathic arthritis (JIA) is a heterogeneous pathology with uncertain causative factors and prognosis, stemming from an immune system dysfunction with the development of autoimmune reactions. The most distinctive and potentially most severe of these is systemic JIA (sJIA), a disease characterized by sharp rises in temperature and rash. A thorough understanding of the complex of immune regulatory mechanisms along with genetic analysis reveals complex relationships between autoimmune reactions and auto inflammation. Sequencing of 15 auto inflammatory genes was performed in 62 patients with JIA: 26 – oligoarthritis, 20 – polyarthritis, 16 – systemic. Studies have shown that 16 (25.8%) patients with the clinical JIA phenotype had changes in nucleotide sequence in the genes encoding auto inflammatory immune response proteins. NOD2 changes were in 12 (19.3%) of them and 1 change in each of the 4 patients NLRP12 (heterozygote, c.1343G> C (p.Gly448Ala)), MEFV (pathogenic heterozygous, c.2082G> A (p.Met694Ile)), ADA2 (heterozygote, c.145C> T). Arg49Trp)), PSTPIP1 (heterozygote, c.806T> A (p.Ile269A)) in the group of studied children with JIA. The study will allow identifying individual genetic loci of JIA risk, expand understanding of the pathogenesis and spectrum of phenotypic manifestations of the disease, improve diagnosis and prediction of its course, as well as reveal new opportunities for monitoring patients with JIA and their personalized therapy.

Keywords: children, arthritis, genes, autoinflammatory.

I. Introduction

In modern rheumatology, juvenile idiopathic arthritis (JIA) is defined as a heterogeneous pathology of uncertain aetiology, which is based on the immune system dysfunction along with the formation of autoimmune reactions [Ringold S. and Angeles-Han S.T., 2019]. The existing criteria for the classification of JIA are based on the phenotypic, genetic and molecular heterogeneity of the disease, which requires further pathogenesis studying [Martini A. and Ravelli A, 2019]. Rheumatological tests are often negative in clinical symptomatology of JIA, and children without clinical symptomatology of arthritis may have false-positive specific antibodies, which contributes to the difficulty in diagnosis. Modern treatment technologies do not always make it possible to achieve the desired effect since the mechanisms of molecular remission and the ability to restore immunological tolerance remain unclear; sustained remission is achieved infrequently, requiring long-term pharmacological therapy without reliable predictive biomarkers for therapy response [Savic S, Wilson AG, et al, 2017]. Currently, there is increasing evidence that the concept of "juvenile idiopathic arthritis" involves a number of clinical and immunological syndromes with different development mechanisms, prognosis and therapeutic approaches [Savic S , Wilson , et al, 2017].

Recent studies indicate an important role of heredity in initiating autoimmune responses in JIA. The increase in the prevalence of familial risk to 40-50% among seropositive arthritis (especially in first degree relatives) is determined by the presence of a set of allelic genes in the HLA-DRB1 locus; the expression of these genes results in production of proteins on the membrane of immune cells containing "shared epitope" [Frisell T.,2016;Guseva V., 2019]. However, HLA genes determine only 17.0% to 56.0% of heredity, so the search for candidate genes is constantly expanding [Hollenbach J.A, 2010].

A genome-wide association studies in the GWAS study has identified 377 genes in 100 risk loci based on data from 29,880 patients with rheumatoid arthritis (RA); 98 of them were associated with a twofold increase in risk of its development, and 15 were identical to immunodeficiency syndromes, involved in the regulation of inflammatory processes, including caspase-8 and -10, autoimmune regulator (AIRE), IL-2 receptor α (CD25), protein tyrosine phosphatase receptor type C (CD45), protein 1, which activates VDJ recombination (RAG1), RAG2, CD40, serine-protein kinase ATM, non-receptor tyrosine-protein kinase TYK2 (TYK2), IFNγ-2 receptor, interferon regulatory factor 8, NFKB, TLR4 and others. [McAllister and Orozco G., 2011].

Mutations in the *TNFRSF1A, NLRP3*, *MEFV*, *NOD2* genes, etc, which determine the inflammatory processes due to abnormal activation of the innate immune system without the synthesis of highly active auto antibodies and antigen-specific T and B cells (synthesis of signaling proteins, interleukins, cytokines, TNF, NOD-like receptors) associated with arthritis, are conventionally allocated to a separate group of systemic auto inflammatory diseases (SAIDs). However, there is

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increasing evidence that innate immunity plays a significant role in the pathogenesis of autoimmune diseases, including JIA [Angelotti F., 2017]. We have also noticed that some patients with juvenile arthritis have symptoms as patients with systemic auto inflammatory disorders have (fever, severe joint swelling, erythematous rash, increased ESR, C-reactive protein). These patients do not respond or do not respond adequately to protocol therapy, have frequent aggravations and an intermediate prognosis. Identification of such patients from a heterogeneous group of children with arthritis for personalized therapy is an urgent task of modern Rheumatology.

Objective: to determine the features of the clinical progression of JIA on the background of changes in the nucleotide sequence of innate immunity genes.

II. Materials and Methods

Studies were conducted in 62 children diagnosed with JIA from 1 to 17 years (27 boys, 35 girls), who were observed in the Department of Connective Tissue Diseases of the Institute. The average duration of the disease was (4.3±3.3) years. The diagnosis of JIA has been established according to the criteria of the International League of Associations for Rheumatology, ILAR [Ringold S., 2020]. Genetic changes have been verified and decoded in the ExAC genetic database(A MedGen ID is a unique identifier referring to an article in MedGen, NCBI's centralized database of information about genetic disorders and phenotypes. Search by MedGen ID at http://www.ncbi.nlm.nih.gov/medgen. An OMIM number is a unique identifier referring to a comprehensive entry in Online Mendelian Inheritance of Man (OMIM). Search by OMIM number at http://omim.org/)

Parents of patients under 12 years and patients over 12 years have received informed consent for the examination and have provided written consent. The study was performed in compliance with the provisions of the GCP (1996), the Convention on Human Rights and Biomedicine (04.04.1997), the World Medical Association Declaration of Helsinki (1964-2002), the Order of the Ministry of Health of Ukraine № 281 dated 01.11.2001.

Patients were stratified by clinical subtypes of JIA: 26 (41.9 %) patients with oligoarthritis, 20 (32.2 %) with polyarthritis, 16 (25.8 %) with systemic arthritis. 19 (30.6%) children were positive for HLA-B27-antigen (JIA enthesitis-related, HLA-B27-positive – JIA-B27), and 4 (6.4%) children were with anterior uveitis (JIA-uveitis). At the onset of the disease, 35 (56.4 %) children had increased acute phase indicators (stage II activity). All children were negative for rheumatoid factor (RF) and did not have antibodies to citrullinated vimentin (A-CCP), 35 (56.4%) children were positive for antinuclear factor (ANF+) (table 1). 4 (6.4%) patients were diagnosed with selective IgA immunodeficiency.

Table 1: General characteristics of patients with JIA

Indicator	Indicator values
Number of patients, abs.number	62
Age, years Me	9 (1-17)
Duration of illness, years	(4,3±3,3)
Gender (boys/girls), abs.number	27/35
JIA subtype, abs.number (%): Oligoarthritis JIA Polyarthritis JIA Systemic JIA	 26 (41,9) 20 (32,2) 16 (25,8)
ANA (+), abs. n. (%)	35 (56,4)
ANA (-), abs. n. (%)	27 (43,5)
HLA-B27 (+), abs.n. (%)	19 (30,6)
HLA-B27 (-), abs. n. (%)	43 (69,3)
ACCP (-),abs. n (%)	62 (100)
RF (-),abs. n (%)	62(100)
Selective IgA deficiency, abs. n (%)	4 (6,4)

ANA-antinuclear antibodies, IgA-immunoglobulin A; HLA-B27-Human leukocyte antigen B27; ACCP- anti-citrullinated protein antibody; RF - rheumatoid factor

For the genetic sequencing, a sample of the condensed epithelium of the oral cavity was taken into a Saliva Collection Kit Oragene TM test tube (DNA Genotek Inc.3000-500 Palladium Drive Ottawa, ON, Canada K2V 1C2). A high-performance panel exomic new generation sequencing (NGS), based on the decoding of fragments of the DNA molecule, has been performed on the Illumina's HiSeq device (manufactured in the USA) in the Invitae laboratory (USA), each change in the nucleotide sequence is sequenced by Sanger. The target enrichment was applied to coding gene sequences of auto inflammatory syndromes: NOD2, NLRC4, NLRP12, NLRP3, PLCG2, MEFV, ADA2, ELANE, LPIN2, MVK, NLRC4, PSMB8, PSTPIP1, TNFRSF1A, TRNT1.

III. Results

The results have shown that 16 (25.8 %) patients with the clinical JIA phenotype had changes in the nucleotide sequence in the genes encoding auto inflammatory immune response proteins, of which 12 (19.3%) children had changes in the NOD2 [c.2104C>T (p.Arg702Trp)] This sequence change replaces arginine with tryptophan at codon 702 of the NOD2 protein (p.Arg702Trp). The arginine residue is moderately conserved and there is a moderate physicochemical difference between arginine and tryptophan. This variant is present in population databases (rs2066844, ExAC 3%), including multiple homozygous individuals. Numerous population-based case-control studies have shown that this variant confers an elevated risk of Crohn's disease [Peter I, Mitchell AA,2011; Hradsky O, 2008; Naderi N., 2011; Lakatos PL., 2005; Tukel T.,

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2004] In a large meta-analysis involving 75 case-control studies with 18,727 cases and 17,102 controls [Yazdanyar S., 2009], individuals carrying this variant had an increased overall risk of Crohn's disease (OR = 2.2, 95% CI 2.0-2.5). (Table 2).

Table 2: Auto inflammation candidate genes in juvenile idiopathic arthritis

Gene	Variant	Zygosity	Variantclassification
NOD2	c.2104C>T (p.Arg702Trp)	heterozygous	IncreasedRiskAllele
ADA2	c.145C>T (p.Arg49Trp)	heterozygous	UncertainSignificance
NLRP12	c.1343G>C (p.Gly448Ala)	heterozygous	UncertainSignificance
PSTPIP1	c.806T>A (p.Ile269Asn)	heterozygous	UncertainSignificance
MEFV	c.1341G>C (p.Lys447Asn)	heterozygous	UncertainSignificance

When all three NOD2 genotypes were combined (p.Arg702Trp, p.Gly908Arg, and p.Leu1007Profs*2), the odds ratios for Crohn's disease were 2.4 (95% CI, 2.0-2.8) for simple heterozygotes, 9.0 (95% CI 6.0-13.5) for compound heterozygotes, and 6.7 (95% CI 4.1-10.9) for homozygotes, compared with noncarriers. This variant is also referred to as R675W and SNP8 in the literature. ClinVar contains an entry for this variant (Variation ID: 4693). Experimental studies have shown that this missense change results in decreased NFkB activity and decreased response to lipopolysaccharide, muramyl dipeptide, and peptidoglycan compared to wildtype protein [Bonen DK., 2003; Li J, Moran T., 2004; Stevens C, 2013]. In summary, this is a frequently observed variant that is associated with approximately a 2.2-fold increased risk of Crohn's disease in population studies. Therefore, it has been classified as an Increased Risk Allele. and one mutation in 4 patients – in genes NLRP12 [c.1343G>C (p.Gly448Ala)]. This sequence change replaces glycine with alanine at cod on 448 of the NLRP12 protein (p.Gly448Ala). The glycine residue is highly conserved and there is a small physicochemical difference between glycine and alanine. This variant is present in population databases (rs104895566, ExAC 0.01%). This variant has been reported in an individual affected with chronic NLRP12-autoinflammatory disorder [Vitale A., 2013]. ClinVar contains an entry for this variant (Variation ID: 97886). Algorithms developed to predict the effect of missense changes on protein structure and function do not agree on the potential impact of this missense change (SIFT: "Tolerated"; PolyPhen-2: "Possibly Damaging"; AlignGVGD: "Class C0"). In summary, the available evidence is currently insufficient to determine the role of this variant in disease. Therefore, it has been classified as a Variant of Uncertain Significance, MEFV [c.1341G>C (p.Lys447Asn)]. This sequence change replaces lysine with asparagine at codon 447 of the MEFV protein (p.Lys447Asn). The lysine residue is highly conserved and there is a moderate physicochemical difference between lysine and asparagine. This variant is present in population databases (rs756322372, ExAC 0.003%). This variant has not been reported in the literature in individuals with MEFV-related conditions. ClinVar contains an entry for this variant (Variation ID: 279849). Algorithms developed to predict the effect of missense changes on protein structure and function output the following: SIFT: "Deleterious"; PolyPhen-2: "Benign"; Align-GVGD: "Class C0". The asparagine amino acid residue is found in multiple mammalian species, suggesting that this missense change does not adversely affect protein function. These predictions have not been confirmed by published functional studies and their clinical significance is uncertain. In summary, the available evidence is currently insufficient to determine the role of this variant in disease. Therefore, it has been classified as a Variant of Uncertain Significance, ADA2 [c.145C>T (p.Arg49Trp)] This sequence change replaces arginine with tryptophan at codon 49 of the ADA2 protein (p.Arg49Trp). The arginine residue is weakly conserved and there is a moderate physicochemical difference between arginine and tryptophan. The frequency data for this variant in the population databases is considered unreliable, as metrics indicate poor data quality at this position in the ExAC database. This variant has been observed in an individual affected with Behçet's disease [Burillo-Sanz S., 2017]. ClinVar contains an entry for this variant (Variation ID: 375246). Algorithms developed to predict the effect of missense changes on protein structure and function do not agree on the potential impact of this missense change (SIFT: "Deleterious"; PolyPhen-2: "Benign"; Align-GVGD: "Class C0"). In summary, the available evidence is currently insufficient to determine the role of this variant in disease. Therefore, it has been classified as a Variant of Uncertain Significance. and PSTPIP1 [c.806T>A (p.Ile269Asn)] This sequence change replaces isoleucine with asparagine at codon 269 of the PSTPIP1 protein (p.Ile269Asn). The isoleucine residue is highly conserved and there is a large physicochemical difference between isoleucine and asparagine. The frequency data for this variant in the population databases is considered unreliable, as metrics indicate poor data quality at this position in the ExAC database. This variant has not been reported in the literature in individuals with PSTPIP1-related conditions. Algorithms developed to predict the effect of missense changes on protein structure and function are either unavailable or do not agree on the potential impact of this missense change (SIFT: "Deleterious";

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PolyPhen-2: "Probably Damaging"; Align-GVGD: "Class C0"). In summary, the available evidence is currently insufficient to determine the role of this variant in disease. Therefore, it has been classified as a Variant of Uncertain Significance. (*Table 2*)

All examined patients had a burdened family history (missed miscarriages, autoimmune diseases in first degree relatives), frequent bacterial (*Campylobacter, Citrobacter, Escherichia, Helicobacter, Pseudomonas, Staphylococcus, Yersinia*) and/or viral infections during their lifetime. 80.0% of children had an early onset of joint syndrome (in the first 3-4 years of life) with varying degrees of activity and/or frequent relapses. Tothisday, JIA isconsidered a polygenicautoimmune disease with a heterogeneous clinical pattern, without defined clear bio markers of the disease and in sufficient response to existing therapies (nonsteroidalanti-inflammatory drugs, gluco corticosteroids, biologictreatment), associated within ability to obtains table clinical laboratory remission. Searching for new genetic changes not only ingenes associated with adaptive immunity, but also in innateimmunegenes, which are characterized by changes in the functions of the corresponding protein sencoded by the corresponding auto inflammatory genes (NOD2, NLRP12, MEFV, ADA2 and PSTPIP1) is of great interest. This paper presents studied mutations in 32 genes of auto inflammation in children with different phenotypes of JIA (n = 62), which were obtained from 62 children under thestudy.

Mutations in auto-inflammatory genes have been detected in 9 (56.2 %) children who were negative for ANF (-) and in 7 (43.7%) ANF (+) positive children. There were more HLA-B27(-) negative patients in this group than positive ones: 10 (62.5 %) and 6 (37.5%), respectively.

Non-steroidal anti-inflammatory drugs (NSAIDs) therapy was ineffective for this children and positive results were obtained when the anti-inflammatory effect was increased due to gluco corticoids (GCs). All children received basic disease-modifying therapy, which did not allow them to get a stable clinical and laboratory remission, which was manifested in a slow progression of the pathological process.

4 patients (25,0%) with systemic JIA, 3 (18,75%) patients with polyarthritis and 9 (56,2%) patients with oligoarthritis had changes in the nucleotide sequence in auto inflammatory genes. The data obtained indicate that in oligoarthritis changes in the innate Immunity functioning are observed more often compared to the systemic form and polyarticular form of JIA. Mutations in auto inflammatory genes were detected in 6 (37.5 %) children negative for ANA and HLA-B27 and in 4 (25.0 %) children positive for ANA and HLA-B27 [OR=0,55;CI (0,12-2,53)].

Four children with systemic JIA and eight with oligoarthritis had heterozygous changes in NOD2: c.2104C> T (p.Arg702Trp). It is known that NOD2 gene mutations are classically relatable to an increased risk of Crohn's disease (PMID: 19713276), autosomal dominant Blau syndrome (MedGen UID: 348835). The most common NOD2 variants are: c.2104C> T (p.Arg702 Trp), c.2722G> C (p.Gly908Arg) and c.3019dupC (p.Leu1007Profs*2). But none of our patients at the time of the examination had clinical and laboratory data that indicated the presence of inflammatory bowel disease.

NOD2 gene polymorphism exists in 1-3% of the population, which increases the risk of clinical manifestation 2-4 times in heterozygous and 7-9 times in homozygous conditions. 19.3% of patients with JIA have changes in the nucleotide sequence in the NOD2 gene, which determines a statistically significant difference with the population frequency [OR=11,76, CI (2,53-54,59), p<0,001]. This sequence change replaces arginine with tryptophan at cod on 702 of the NOD2 protein (p.Arg702Trp). The arginine residue is moderately conserved and there is a moderate physicochemical difference between arginine and tryptophan. This variant is present in population databases (rs2066844, ExAC 3%), including multiple homozygous individuals.

Numerous population-based case-control studies have shown that this variant confers an elevated risk of Crohn's disease [Peter I, Mitchell AA,2011; Hradsky O, 2008; Naderi N., 2011; Lakatos PL., 2005; Tukel T., 2004]. In a large meta-an alysis involving 75 case-control studies with 18,727 cases and 17,102 controls [Yazdanyar S., 2009], individuals carrying this variant had an increased overall risk of Crohn's disease (OR = 2.2, 95% CI 2.0-2.5). When all three NOD2 genotypes were combined (p.Arg702Trp, p.Gly908Arg, and p.Leu1007Profs*2), the odds ratios for Crohn's disease were 2.4 (95% CI, 2.0-2.8) for simple heterozygotes, 9.0 (95% CI 6.0-13.5) for compound heterozygotes, and 6.7 (95% CI 4.1-10.9) for homozygotes, compared with noncarriers. This variant is also referred to as R675W and SNP8 in the literature. ClinVar contains an entry for this variant (Variation ID: 4693). Experimental studies have shown that this missense change results in decreased NFkB activity and decreased response to lipopolysaccharide, muramyl dipeptide, and peptidoglycan compared to wild type protein [Bonen DK., 2003; Li J, Moran T., 2004; Stevens C, 2013] . In summary, this is a frequently observed variant that is associated with approximately a 2.2-fold increased risk of Crohn's disease in population studies. Therefore, it has been classified as an Increased Risk Allele.

A 2-year-old girl of Arab origin is being observed in the children`s connective tissue disorder clinic. She was born in 5th pregnancy, complicated by toxicosis and the threatened miscarriage; the previous 2 pregnancies ended in miscarriages, 2 children were born, one of them died at the age of 1 month because

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of congenital heart defect. The girl has been ill since the age of 10 months, when a long-term fever, anemia (Hb 70-100 g/l), increased ESR (25-40 mm/h) and CRP (24-31 mg/l) occurred HLA-B27 (+) , АНФ - 1:1000. She received a course of antibacterial therapy, NSAIDs, which was ineffective. The character of disease was progressive, often recurrent with bouts of fever, arthralgias and edema of the knee, ankle, and wrist joints. Ultrasonography has revealed exudative and proliferative changes in the joints. The girl was diagnosed with juvenile idiopathic polyarthritis. Against the background of GCs therapy and methotrexate, a positive dynamics was obtained, but when the dose of GCs was reduced, the inflammatory process activated, which required the intensification of therapeutic measures.

Genetic sequencing revealed a heterozygous change in the ADA2 gene c.145C>T (p.Arg49Trp), which encodes the adenosine deaminase 2 enzyme. This sequence change replaces arginine with tryptophan at the codon unit 49 of the ADA2 protein (p.Arg49Trp).

The onset of systemic JIA in a girl was at the age of 8 years and was characterized by fever, skin rash, arthritis of the knee and hip joints, and myositis. Laboratory tests showed an increase in CRP (24-20 mg/l), ESR(68-20 mm/h), CPK (7086-145 U/l), LDH (707-645 U/l), ANA 1:1000, negative RF, HLA-B27 (+). In the treatment with systemic GCs, hydroxychlorhynine and methotrexate, positive dynamics were obtained, but with a decrease in GCs therapy, there is a constant disease recurrence, which required changes in the baseline therapy and initiation of biological immunotherapy.

Genetic sequencing revealed a pathogenic mutation in the NLRP12 gene. Mutations in the NLRP12 gene determine the activation of NF-kB and caspase signaling pathways and are associated with autosomal dominant Familial cold autoinflammatory syndrome (FCAS) (MedGen UID 435869). The clinical significance of this variant remains uncertain. The sequence change replaces glycine with alanine at the 448 NLRP12 protein codon (p.Gly448Ala). This variant has been reported in a person suffering from chronic NLRP12 inflammation [Naderi N., 2011]. ClinVar contains an entry for this variant (variant ID: 97886). The available evidence is still insufficient to determine the role of this variant in the disease. Just as in case with FCAS, patients with NLRP12 experience periodic episodes of high temperature under the influence of cold, which last for 2-10 days every 3-4 weeks. Fever is usually followed by arthralgia, myalgia, abdominal pain, headache, lymphadenopathy, aphthae on the oral mucosa and skin rash.

In a 3-year-old boy, the disease debuted at the age of 1.5 years, when a spotty rash appeared at the height of the temperature, arthralgia, arthritis of the right knee joint. Systemic JIA was established and GCs therapy, methotrexate were prescribed; it gave a short-term positive effect, but after the third administration there was an allergic reaction, so that the therapy was replaced with the administration of tocilizumab, which allowed to get a positive dynamics and cancel the GCs administration. After a year rash had gradually re-appeared in child; it spread all over the body; the GCs were prescribed again with a positive result.

Genetic testing revealed a change in the nucleotide sequence in the PSTPIP1 gene (MedGen UID: 346801), which encodes a cytoskeletal protein. This protein is active in interaction with various inflammatory proteins; it binds to the cytoplasmic tail of CD2, the t-cell activation and adhesion effector, binds PEST type protein tyrosine phosphatases and directs them to c-Abl kinase to mediate c-Abl-dephosphorylation, thereby regulating the activity of c-Abl. It also interacts with pyrin, which is found in association with the cytoskeleton in myeloid/monocyte cells and modulates immunoregulatory functions. Mutations in this gene are associated with PAPA syndrome (Pyogenic Arthritis, Pyoderma gangrenosum and Acne) and disrupt the physiological signaling necessary for maintaining proper inflammatory response. The genetic defect leads to increased binding of pyrin to NLRP, which causes autoinflammatory reactions [Gupta V., 2018]. The clinical significance of the identified variant is uncertain. This sequence change replaces isoleucine with asparagine in the codon 269 of PSTPIP1 protein (p.Ile269Asn). The isoleucine residue is highly conserved and there is a large physical and chemical difference between isoleucine and asparagine.

IV. Discussion

During genetic testing, 25.8 % of patients with JIA (with ILAR/ACR diagnostic criteria) were found to have mutations in the NOD2, NLRP3, ADA2, MEFV, and PSTPIP1 genes, which increases the risk of initiating auto inflammatory syndromes characterized by arthritis, recurrent episodes of inflammation, fever, etc. [Shnappauf O and Aksentijevich, 2019].

This paper did not aim to determine the distribution of all eles in genes at the population level, but only in a limited cohort of children with different JIA phenotypes. Therefore, follow-up studies in this regard are appropriate.

It is difficult to correct the individual clinical phenotypes of JIA that we have presented with protocol treatment, which indicates the possibility of the existence of juvenile arthritis with genetic variants of auto inflammatory syndromes and disorders in certain functions of innate immunity: a burdened family anamnesis of autoimmune diseases in first degree relatives, acute onset of arthritis after bacterial or viral infections, prolonged fever, rash. It should be noted that these children did not have RF and ACCP, and most of them were HLA-B27 negative.

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In 19.3 % of children, there were variation changes in the nucleotide sequence in the NOD2 gene, which mutations are classically associated with Crohn's disease, Blau syndrome signs of which were not detected in our patients. A significant difference in the frequency of NOD2 mutations in our patients with JIA compared to the population indicates the significance of this genetic variation in onset initiation of juvenile arthritis, the mechanisms of which have not yet been fully determined [ExAC Database; Negroni A., 2018]. The NOD2 gene encodes a protein that plays an important role in the activities of cells of both the innate and adaptive immune system (including monocytes, macrophages, and dendritic cells). It happens through the regulation of cytokines, chemokines, and antimicrobial peptides that take part in the antibacterial and antiviral response. NOD2 protein is also active in certain types of epithelial cells, including Paneth cells, in the intestinal mucosa; it is also involved in the recognition of bacteria and modulates the protective function of the immune system of the mucous membranes. NOD2 protein takes part in such processes as autophagy, apoptosis and proteolysis, determines innate inflammatory responses to bacteria and viruses through the activation of NF-KB and caspase-1 pathways, which leads to increased expression of pro inflammatory factors, including IL-1β, TNF-α, IL-6, IL-12p40, IL-8, chemokine ligands, antimicrobial factors.

In addition to its main role in the innate immunity, NOD2 is able to activate the adaptive immune system. It is a key regulator of T-helper 2 cells, which leads to the expression of IL-4 and IL-5. Several studies have shown that joint stimulation by NOD2 agonists and TLR receptors (TLR) causes synergistic production of Th1-associated cytokines in various cell types, although the mechanisms of such reactions are unknown. NOD2 activation, in addition, contributes to the formation of Th17 cells and the production of IL-17A, IL-17F, IL-21 and IL-22 [Brembilla NC, 2018].

The involvement of NOD2 in the pathogenesis of various genetic diseases indicates that this protein is a key regulator of immune and inflammatory responses and plays a crucial role in maintaining the balance between bacteria, epithelium, and the innate immune response of the organism. This protective function is absent in case of NOD2 mutation, which leads to an exacerbation of inflammation and the clinical manifestation of various diseases. Many questions remain unanswered, including the relationship between NOD2 mutations and the microbiota as well as understanding the processes through which mutations in NOD2 can be associated with susceptibility to inflammation and the development of diseases.

Recent studies indicate that the NOD2 gene can be activated in idiopathic arthritis with increased production of TNFa, IL-8, and IL-1β by peripheral blood mononuclear cells, while decreased NOD2 regulation reduced the level of proinflammatory cytokines, NF-kB, TRAF6, and IKK [Franca RFO, 2015]. NOD2 protein is expressed in fibroblasts and synovial fluid of patients with RA [Gupta V, 2018]. It is supposed that the NOD2 regulatory pathway is functional, since stimulation of peripheral blood mononuclear cells with muramyl dipeptide (MDP) has induced the production of larger amount of tumor necrosis factor (TNF-α), interleukins (IL-8 and IL-1β) compared to osteoarthritis. Synovial fluid obtained from patients with RA is able to activate the NF-kB signaling pathway [Kim HW, 2017].

Therefore, activation of NOD2-associated auto inflammatory mechanisms can lead to modification and transformation of autoimmunity, which should be taken into account for treatment of juvenile arthritis. Elucidating the mechanisms of regulation and function of NOD2 in juvenile arthritis may lead to the development of an effective therapeutic strategy for inflammation.

The change in the nucleotide sequence in the ADA2 gene in a child with idiopathic arthritis calls attention to itself too. To date, more than 60 mutations of this gene have been identified; they cause changes in the adenosine deaminase 2 enzyme, the deficiency or activity loss of which is characterized by abnormal inflammation of organs and tissues with various clinical phenotypes, including vasculopathy, stroke, hematological and multisystemic disorders that can occur along with arthritis. An enzyme deficiency is assumed to be able to disrupt the balance between proinflammatory and anti-inflammatory macrophages in tissues and lead to the accumulation of proinflammatory macrophages resulting in abnormal inflammation [Lee PY, 2020]. ADA is considered a probable candidate gene for susceptibility to common immune-mediated diseases. ADA polymorphism in the clinical modification of RA and response to methotrexate treatment has been less studied [Lee PY, 2020].

The mutation found in the child in our study does not correspond to an autoinflammatory disease, which requires further research for the possibility of new prospects for the treatment of this category of patients. Frequency data for this variant in population databases is considered unreliable, and the ExAC database indicates poor data quality in this position. The variant was registered in a person with Behcet's disease [Burillo-Sanz S., 2017]. ClinVar contains an entry for this variant (option ID: 375246).

The protein, which is encoded by NLRP12, inhibits the activation of nuclear transcription factor NF-kB, which regulates the expression of proinflammatory cytokine genes, activates caspase 1. Inadequate functional activity of NLRP12 can lead to the development of cryopyrin-associated periodic syndrome (CAPS) [Gupta V., 2018]. In our patient, who has changes in the nucleotide sequence in this gene, the clinical picture does not correspond to CASP, but

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requires differential diagnosis between systemic JIA and systemic connective tissue diseases.

Our data correspond to the results obtained during the study, involved a large group of patients from the UK, which has also found associations between psoriatic JIA and mutations in NLRP3, NOD2, MEFV and PSTPIP1 [Brembilla NC, 2018]. These data confirm the significance of extrapolation from monogenic syndromes to identify candidate genes for susceptibility to such a complex disease as JIA.

Thus, mutations of four genes that are presented in our work encode proteins that are the main protective components of the innate immune system against intracellular pathogens, and increase the secretion of IL-1β by activating caspase 1. Perhaps, polymorphisms in the studied genes may have an effect on the expression of IL-1β, and subsequently affect the onset of JIA. In case if our findings are confirmed in other studies, we will be able to provide evidence for the advisability of treatment of specific variants of JIA with IL-1β antagonists. However, the sample size in this cohort was too small for adequate results verification, and further studies with a larger cohort of JIA patients are required to confirm them.

V. Conclusion

Nucleotide sequence changes in NOD2, NLRP3, MEFV and PSTPIP1 genes were detected in 25.8% of patients with JIA, whose clinical phenotypes are characterized by arthritis with recurrent episodes of inflammation, fever, rash, lackof RF, ACCPand who are more often HLA-B27 negative, are poorly treated by protocol therapy, which determines the possibility of the existence of juvenile arthritis with variation sininnate immunity genes.

In 19.3% of children, variable changes in the nucleotide sequence in the NOD2 gene were detected. A significant difference in the frequency of NOD2 mutations in patients with JIA compared with the population indicates the importance of this genetic variation for the initiation of juvenile arthritis.

Therefore, insomecases, juvenil earthritis may have a mixed clinical phenotype of auto immune-auto inflammatory overlap, which should be considered when choosing a personalized therapeutictactic.

JIA is a relatively rare pediatric disease, in which few full-genome studies have been conducted, especially at the population level. Therefore, follow-up research in this regard is appropriate.

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Petrological, Geochemical and Mechanical Characteristics of Kooz Kunarr Khewa Calcite Marble

By Ahmad Faham Bawary, Safiullah Noorzai, Usman Amin, Sohaib Nader, Ulfatullah Noori, Hamid Zaheer & Abdul Haq Salih

Abstract- This methodical investigation's primary goal is to ascertain the geological, geochemical, mineralogical, and mechanical characteristics of the calcite marble found in Khewa. The article includes all important information about Khewa calcite marble, including its quality, constituent minerals, related elements, and other data. The procedures employed for the precise investigation and evaluation of the mine's characteristics include manipulative field observation, laboratory examination of Khewa calcite marble, and preliminary library studies concerning the research location. The calcite marble library research provided exact and accurate information about the location of the examined area's geology, as well as information about its tectonics and magmatism. In order for the laboratory analysis to determine the true geology of the area under study, including adjacent rocks, and the chemical makeup of the calcite marble, laboratory examination is required. Mica schist is the neighboring rock to calcite marble (a metamorphic rocks).

Keywords: *marble, calcite marble, tectonic, XRF, khewa, nangrhar.*

GJSFR-G Classification: *FOR Code: 040399*



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Petrological, Geochemical and Mechanical Characteristics of Kooz Kunarr Khewa Calcite Marble

Ahmad Faham Bawary [α], Safiullah Noorzai [σ], Usman Amin [ρ], Sohaib Nader [Ω], Ulfatullah Noori [¥], Hamid Zaheer [§] & Abdul Haq Salih [χ]

Abstract- This methodical investigation's primary goal is to ascertain the geological, geochemical, mineralogical, and mechanical characteristics of the calcite marble found in Khewa. The article includes all important information about Khewa calcite marble, including its quality, constituent minerals, related elements, and other data. The procedures employed for the precise investigation and evaluation of the mine's characteristics include manipulative field observation, laboratory examination of Khewa calcite marble, and preliminary library studies concerning the research location. The calcite marble library research provided exact and accurate information about the location of the examined area's geology, as well as information about its tectonics and magmatism. In order for the laboratory analysis to determine the true geology of the area under study, including adjacent rocks, and the chemical makeup of the calcite marble, laboratory examination is required. Mica schist is the neighboring rock to calcite marble (a metamorphic rocks). The researcher gathered five random, exact samples of the calcite marble from various locations in the field to acquire this. For the desired outcomes, various techniques were used on the assembled simples. The purpose of XRF, also known as X-Ray fluorescence, is to determine the precise proportion of certain elements and oxides. In addition to the calcite, which is discovered in small amounts in the Mica schist rocks that surround the calcite marble in the XRF results, the percentage of calcite is over 55%. The Mica schist neighboring rocks of the calcite marble also contain a small quantity of uranium. The unique characteristics of calcite marble, such as their hues, structures, and linked minerals, were elaborated by the microscopic analysis. We obtained a range of results from physical and mechanical tests such compressive strength, and unit weigh. Wherever iron, aluminum, and nickel are found, calcite marble is very hard. Overall, Khewa calcite marble is very hard but suitable for construction.

Keywords: marble, calcite marble, tectonic, XRF, khewa, nangrhar.

I. Introduction

Marble is the product of the recrystallization of limestone or dolomite by contact metamorphism or regional metamorphism The mineral composition is mainly calcite, which foams when it encounters hydrochloric acid Marble is also a large category of natural architectural decorative stones. Generally, marble cutting machines refer to metamorphic or unclassified carbonate rocks with decorative functions, which can be made into architectural stones or crafts.

Marble is commonly used as Building Stone, Countertops and sinks, Floor tiles, Terrazzo - marble chips mixed with concrete to form floors, Tomb Stones construction and dimensional stone in Afghanistan especially in eastern part of Afghanistan Nangrhar province such as Khewa Calcite Marble. There are different varieties of marble in different provinces such as Kunarr, Bamyan, Kabul, Balkh, Kandahar, Herat, Paktia, Parwan, Helmand, Nangrhar, Faryab, Wardak, and Samangan. which are extracting and supplying to different countries and are prized for

II. Geology of Study Area

It is necessary to consider the geological structure of this Tectonic Zone. The Tectonic zone of Jalalabad is located in eastern part of Afghanistan and the southeastern part of Nuristan zone. For the first time this was separated by Prof. Slavin and Syed Hasham Mirzad in year (1969) and Kalchanof and Sayed and added this zone in to Tectonic map and relate this zone with Alf Core.

a) Jalalabad Tectonic Zone (Spinghar Block)

The Jalalabad zone is a part of the depth of the past Cambrian which trace minerals lies above the ridges of the past Cambrian that form base of Geosyncline. These ridges appear in the mountain to the surface of the earth. In the eastern part of the Zone and in the southeastern part of Jalalabad city, on the left bank of the Kabul river and on the banks of Kunarr river the upper Paleozoic and lower Mesozoic sediment are visible on the ground and the central part of the Jalalabad zone is covered by Neogene Deposit.

In addition, In Jalalabad zone there are sediments ranging from Archean Proterozoic to Quaternary system. The Archean Proterozoic structure are composed of various Gneisses, Quartzite, Amphibolite and crystalline slabs including the white

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Marble layers of Marble. The thickness of the Marble layers inside the Gneisses is from 300 to 600 meters.

Furthermore, The Paleozoic group formation are relatively extensive in the Jalalabad zone including the Ordovician, Silurian, Devonian and Carboniferous formation. These formations are composed of Quartzite. Sediments, Sandstone and Limestone the relationship between which is not clear in some places. The sediments of the Neogene system are present on the top of the sediment disconcordantly which include Conglomerate Sandstone and Clays. The sediment of Quaternary has filled the shore of the river and their thickness is also high.

Relatively in this zone Magmatic rocks are present in large amount that are visible on the ground of the left of Kunarr river and in the Spinghar mountain range which differ from one another in age and in composition and are divided in to two different complex.

1. Granite, Gabbro, Amphibolite Complex
2. Granite, Granodiorites Complex

And also Albite that are spread in different part of the Zone. And these Magmatic rocks are interesting according to Mines presence.

Tectonically Jalalabad zone is associated with the Cambrian structure with three structural formation layers separated by

O-T, AR-PR and N-Q

In a tectonic map in 1973 the department of Geology and Mines has divided Jalalabad zone to three further sub zone in this region.

1. The Kunarr Tectonic Zone
2. The Spinghar Tectonic Zone
3. The Jalalabad Basin (Waizy, July 2020)

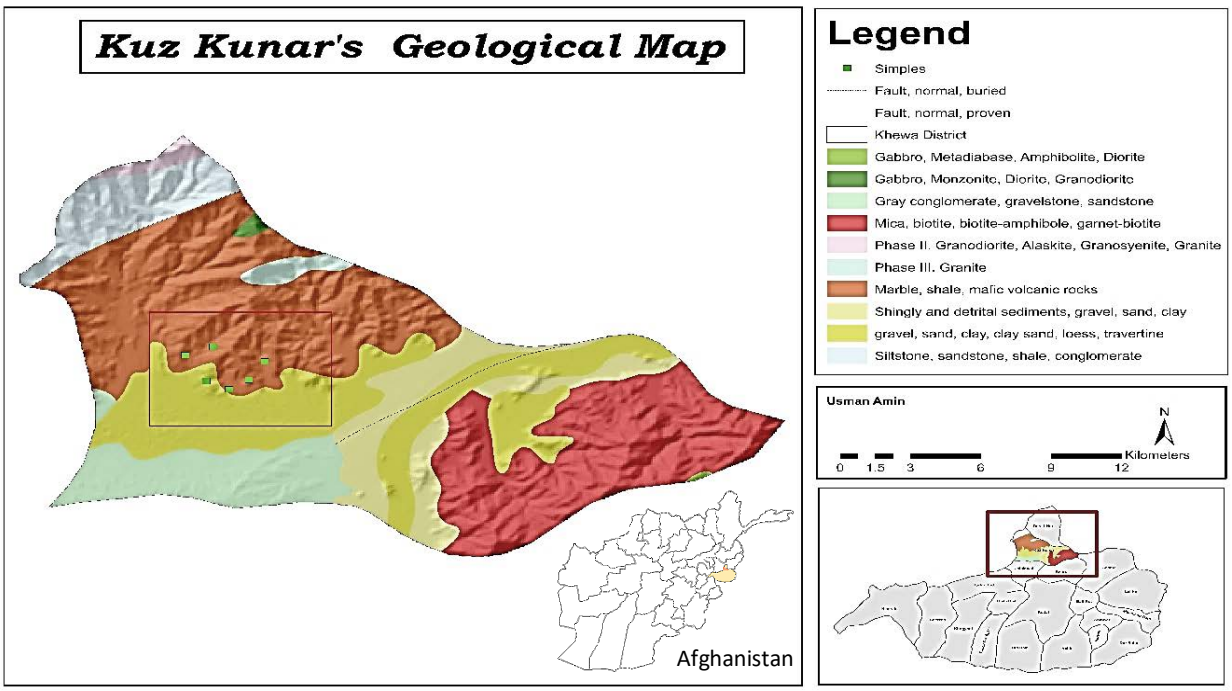

Figure 1: Shows the geological map of the marble area

b) Methodologies

To gather the prehistoric studies of the marble for this research, a desk study was undertaken prior to a field trip and laboratory analysis. Books, other articles, and Wikipedia information were all reviewed. For laboratory analyses, samples from the Marble mine were obtained. After that, the area was thoroughly researched by collecting GPS coordinate, readings of the location and taking samples from the precise mine. The conclusions were drawn by interpreting the fieldwork data and analytical data. A sufficient amount of rock sample was crushed and ground up for geochemical tests, and thin slices were made for microscopic examination in the Ministry of Mine and Petroleum. GIS mapping of the mining area makes up the third section of the stud.

i. *Petrographic description of the Samples*

This specimen contains Calcite, Dolomite and Plagioclase with mosaic structure and metamorphic origin. the dye of the specimen is whitish yellow. (figure a,b)

a. This specimen contains Muscovite, Biotite Garnet and Quartz with schistose structure and metamorphic origin. the dye of the specimen is yellowish. (Figure c,d)
b. This specimen contains Muscovite, Biotite and Quartz and other opaque minerals with

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schistose structure and metamorphic origin. the dye of the specimen is white. (Figure e,f)

c. This specimen contains Dolomite and Calcite with mosaic structure and metamorphic origin. the dye of the specimen is pale yellow. (Figure g,h)

d. This specimen contains Muscovite, Biotite and Quartz with mosaic structure and metamorphic origin. the dye of the specimen is yellowish. (Figure g,h)

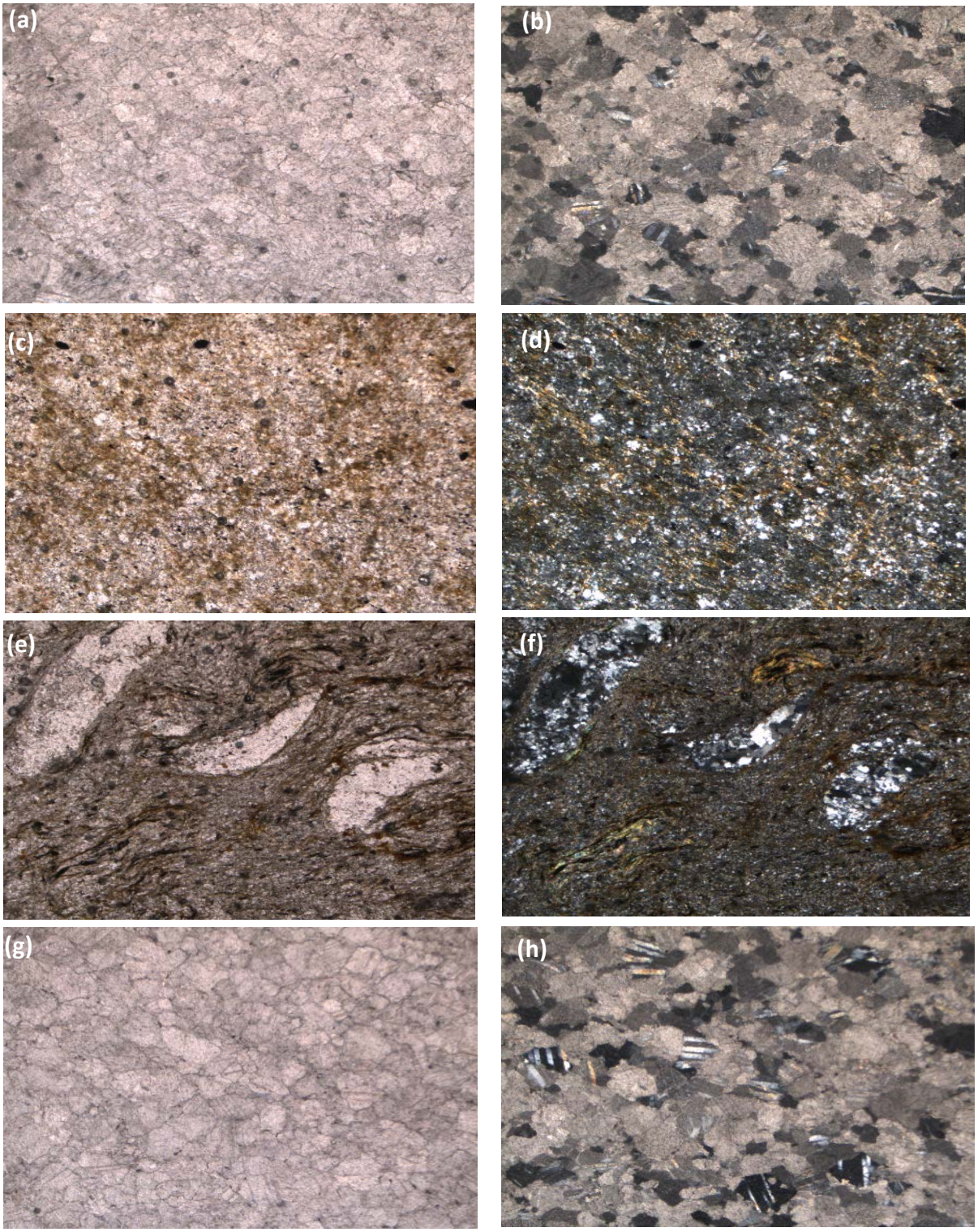

Figure 2: Microscopic Images; (a) Marble PPL and (b)Marble XPL from C1.1 ; (c) Mica schist PPL and (d) Mica Schist XPL, from C2.2 sample; (e) Mica schist PPL and (f) Mica Schist XPL from sampleC3.1; (g) Calcite Marble PPL and (h)Calcite Marble XPL, from sample C4.1

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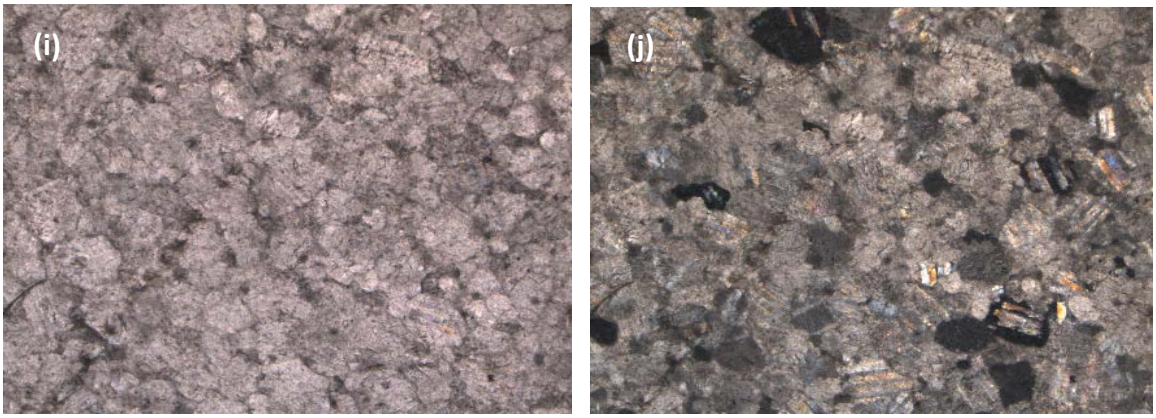

Figure 3: The marble specimen under petrographic microscope,(i) Marble PPL and (j)Marble XPLfrom C5.1 sample

ii. *Geochemical result*

The purpose of geochemical analysis is to identify the constituents of Khewa Calcite Marble's chemical makeup. The XRF analysis results in the determination of 28 elements. The elements with the highest percentages are silicon, calcium, magnesium, aluminum, iron, potassium, and sulfur, as well as heavy metals like Fe, Cr, and Ti.

Table 1: Shows the oxide percentage in Marble in its adjacent rocks with PPM

Oxides	C1-1	C2-2	C3-3	C4-4	C5-5	Over all PPM
MgO	124169.281	129315.227	118810.945	40573.023	41836.957	454705.433
Al_20_3	9800.377	6439.447	40826.293	164967.313	163134.250	385167.680
$Si0_2$	15316.664	11887.871	13935.673	758900.938	90381.525	890422.671
Fe_20_3	385.620	409.443	2564.272	60375.730	52280.992	116016.057
Cr_20_3	101.027	45.940	166.520	254.507	172.603	740.597
CaO	43167.294	41281.397	35411.609	27817.947	24670.201	172348.448
TiO_2	0.000	0.000	762.605	6597.444	6378.446	13738.495
K_20	521.857	382.843	3485.495	32264.771	27118.930	63773.896
$S0_3$	2145.588	2357.083	7541.333	8220.825	4054.696	24319.525
P_20_5	0.000	0.000	0.000	2745.132	3472.462	6217.594

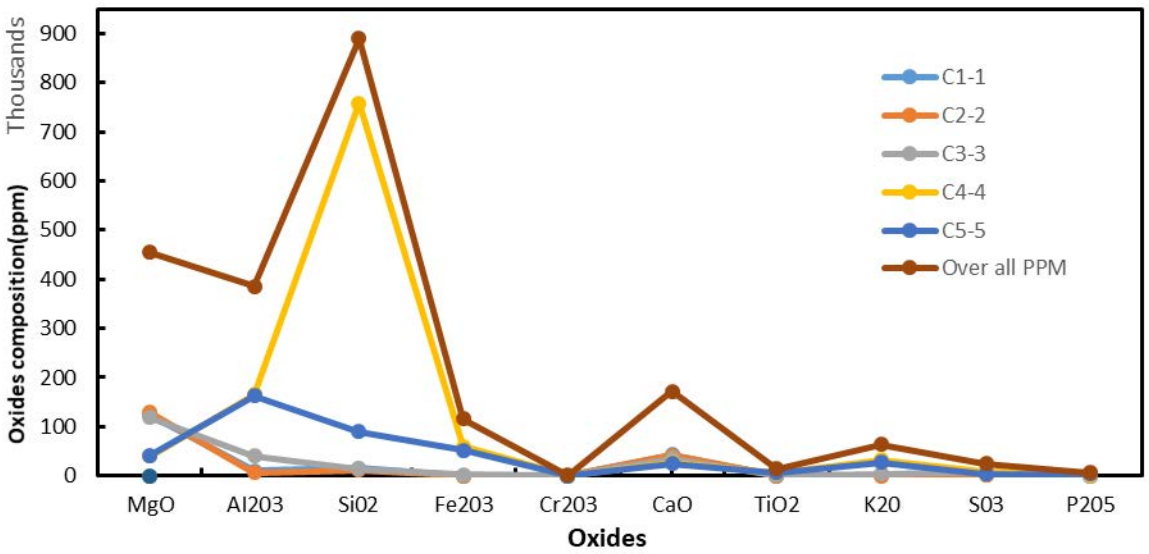

Figure 4: The graph shows the high point of SiO_2

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Table 2: Shows the total 28 elements percentage in Marble in its adjacent rocks with PPM

Elements	C1-1	C2-2	C3-3	C4-4	C5-5	Total PPM	Percentage
Mg	74,800.77	77,900.74	71,572.86	24,441.58	25,202.99	273,918.94	27.3919
Al	5,185.39	3,407.12	21,601.21	87,284.29	86,314.42	203,792.42	20.3792
Si	7,157.32	5,555.08	65,119.97	354,626.59	422,343.56	854,802.53	85.4803
P	0	0	0	1,198.75	1,516.36	2,715.11	0.2715
S	858.235	942.833	3,016.53	3,288.33	1,621.88	9,727.81	0.9728
Cl	415.01	588.91	1,069.03	672.319	520.006	3,265.27	0.3265
K	431.287	316.399	2,880.57	26,665.10	22,412.34	52,705.70	5.2706
Ca	308,337.81	294,867.13	252,940.08	19,869.96	17,621.57	893,636.55	89.3637
Ti	0	0	456.65	3,950.57	3,819.43	8,226.64	0.8227
V	0	43.459	43.082	174.223	143.756	404.52	0.0405
Cr	69.196	0	114.055	174.32	118.221	475.79	0.0476
Mn	125.522	126.257	116.608	395.57	319.658	1,083.62	0.1084
Fe	269.664	286.324	1,793.20	42,220.79	36,560.14	81,130.12	8.1130
Co	0	22.028	0	118.604	0	140.63	0.0141
Ni	60.83	81.547	71.589	94.3	84.973	393.24	0.0393
Cu	0	0	0	49.76	32.263	82.02	0.0082
Zn	15.56	18.8	28.204	91.311	88.923	242.80	0.0243
As	0	0	0	14.258	0	14.26	0.0014
Rb	0	0	0	55.933	52.166	108.10	0.0108
Sr	68.047	50.702	30.84	88.796	100.362	338.75	0.0339
Zr	0	0	0	201.972	178.984	380.96	0.0381
Nb	0	0	0	11.829	11.198	23.03	0.0023
U	0	7.479	0	0	0	7.48	0.0007
Ba	261.008	285.623	218.639	859.116	707.169	2,331.56	0.2332
Au	0	0	0	0	0	0.00	0.0000
Pb	0	9.021	0	23.296	28.11	60.43	0.0060
Bi	0	0	0	15.338	7.112	22.45	0.0022
Th	0	0	0	17.563	7.482	25.05	0.0025

Silicon, Calcium, Manganese, Aluminum and Iron are the elements in the composition of Marble that gave the much hardness, and pale color. In addition, the existence of sulfur with 3.490 percent and prolong chemical weathering have played important role in the alteration of the marble color into pale white color.

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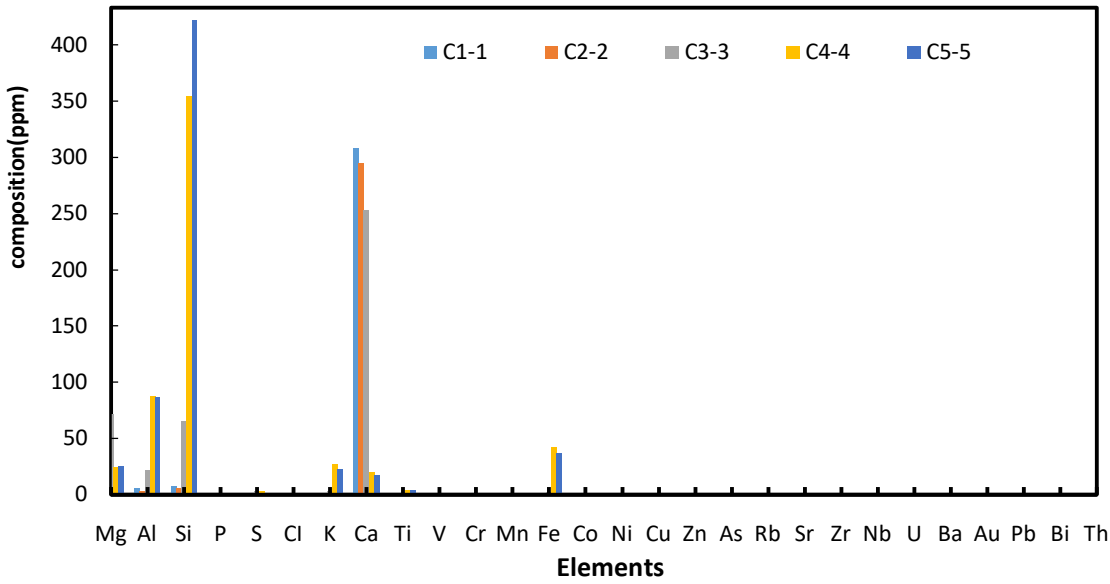

Figure 5: Shows the elements graphs where the Al and Si are in very high range

III. Mechanical Result

The Calcite Marble mechanical studies consist of the following tests which is completely described with their result done in Nangrhar Alfalah University Engineering Laboratory.

a) Compressive Strength

Uniaxial unconfined compressive strength is the amount of load a stone can tolerate before it breaks in to pieces, such measure of stone's ability to support load bearing structures (Prof., 2022)

The table below shows that the uniaxial compressive strength of Calcite Marble ranges from 53 to 81 Mpa under dry to wet circumstances. Based on strength recognition and description, this rating is assigned in the category of powerful rock break with hammer in hand.

Table 3: Shows Compressive strength result of Calcite Marble.

Crushed Aggregate of Khewa Calcite Marble				Material Source	Khewa Calcite Marble
Cube NO	Dimension (mm^2)		Area (mm^2)	Load (KN)	Compressive Strength Kg/Cm^2
	Length	Diameter			
1	71	72	5112	472.5	92.4
2	72	72	5184	278.2	53.7
3	72.1	72.8	5248.88	522.8	99.6
		Mpa		Kg/Cm^2	Psi
		81.9		835.1	11878.11

IV. Unit Weight Test of Calcite Marble

The unit weight test is used to measure the quality of aggregate through determining the placed materials volumes the hallows within the distant range. When the scale of the aggregate reaches to 25(5inch) mm so the mold is used to measure unit weigh. (Prof., 2022).

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Table 4: Shows Compressive strength result of Calcite Marble

Unit Weight of course Aggregate				
Test No		1	2	3
Mass of material + Container	g	4742	4728	4740
Mass of Empty Measure	g	3052	3052	3052
Mass of Material	g	1690	1676	1688
Volume of Measure	Cm^2	928.9	928.9	928.9
Bulk Density	g/cm^2	1.819	1.804	1.817
Average Unit Weight g/cm^2			1.814	

V. Result and Discussion

The results of petrographic investigations performed on constructed simples under a microscope showed that the calcite mineral was present in excess in the majority of the sampled simples, with the Khewa marble having a calcite mineral proportion of exactly 55%. The marble is known as "calcite marble" because to its enormous calcite content. Calcite marble's light white tint is caused by the presence of iron and iron oxide. In addition, the proportions of iron and iron oxides are 8.174% and 11.689%, respectively. Additionally, the inclusion of silicon, titanium, nickel, aluminum, and nickel oxides added to the calcite marble's incredible hardness. Calcite, Dolomite, Plagioclase, Muscovite, Biotite, and Quartz are only a few of the minerals that make up calcite marble that were discovered in petrographic research. The XRF examination identified the elements and oxides that were present in the calcite marble's composition. Si, Ca, Mg, Al, Fe, K, S, Ti, Cl, Ba, P, Mn, Cr, Ni, Sr, V, Zr, Zn, Co, Rb, Cu, Pb, Th, Nb, Bi, As, U, and Au are the twenty-eight elements determined by the XRF with their precise percentages. The composition of calcite marble contains significant amounts of silicon, calcium, magnesium, and aluminum. Along with the calcite marble, eight oxides are also present in addition to the elements. CaO, SiO2, Al2O3, Fe2O3, SO3, K2O, TiO2, P2O5, and Cr2O3 are the mineral oxides. High percentages of MgO, CaO, SiO2, and Al2O3 are present. The presence of uranium with a 0.0007479% concentration in the sample is noteworthy in the XRF examination. Studies in mechanics and physics produced thorough findings. Calcite marble has a compressive strength of 81.9 MPa in dry form and 53.4 MPa in humid form. Physical and mechanical testing showed that Khewa calcite marble is suitable for use in drainage walls and basements but should not be used for interior decorating, kitchen countertops, or other locations where there is a lot of moisture or humidity.

VI. Conclusion

1. Although there was a small amount of uranium in the calcite marble, the XRF analysis elaborated its existence. Carbonate rocks are the primary cause of the existence of uranium.
2. Out of the other twenty-eight elements, silicon, calcium, magnesium, aluminum, and iron were the elements most often detected.
3. The calcite marble now has four gradations of hardness due to the outstanding interplay of silicon, iron, and aluminum.
4. Ten oxides have been collected, but among them, magnesium oxide, calcium oxide, silicon oxide, aluminum oxide, and iron oxide have the highest percentages.
5. Due to the presence of iron and extended physical and chemical weathering, the marble has developed a pale white tint. However, apart from this, the color has also lightened significantly within.
6. Calcite, dolomite, and plagioclase are the three important minerals, whereas quartz and mica (muscovite and biotite) are the accessory minerals, according to the microscopic study. Additionally, mica schist makes up the adjacent rocks.
7. Due to its low water absorption, marble may be used in basements, drainage walls, and particularly in damp areas.
8. According to Los Angeles studies and compressive strength, the marble can withstand a large amount of pressure and weight.

VII. Suggestions

1. Extensive research showed that the tested samples all had a trace level of uranium after the XFR assessment. Because it causes cancer and other harmful diseases, the province authorities must inform the permanent people who live close to the calcite marble mountainous area of the Khewa.
2. Given that we are aware of the superior quality of Khewa marble, we highly advise the present administration to launch a thorough prospecting study to learn more about the locations of calcite marble.
3. To accurately analyze the calcite marble and their internal fractures. Deep drilling is required to figure out whether or not a prospected region may produce blocks of calcite marble that are desirable.

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4. The Khewa district to Kunarr Khaas districts are where calcite marble first began to thrive. We sincerely want the government to produce accurate and comprehensive satellite, regional, topographical, and geological imagery. This will help the government, businesses, and other researchers.
5. In the field of research, calcite marble from the locations that produce good blocks should be utilized for interior decorating of homes, sculptures, and tabletops, while calcite marble from the locations that produce poor blocks should be used for roads, basements, and other sites.
6. The marble extraction process started some years ago. Additionally, tons of marble have been rotting as a result of extractors using outdated equipment. The government must maintain a close eye on upgraded machinery and must force extractors to employ these tools for efficient extraction.
7. To encourage vendors who have been functioning with marble, travertine, and granite to increase their investments in the marble industry, the government should prolong contracts for up to 10 years.
8. The government need to refrain from extracting the calcite marble from areas where nickel, cobalt, and iron concentrations are high.
9. In order to come down with the loss of marble, the business owner needs pay professionals to do the extraction.
10. The government ought to waive or lower the tax in order to encourage marble dealers.
11. The value of marble domestically in the nation will be impacted by illegal marble exports to foreign nations. The Afghan government must stop illicit exports.
12. In order to generate high-quality cement, the government should persuade traders to participate in the cement business.

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Antimeiotic Properties of the Aqueous Extracts of Leaves, Fruits and Roots of the Muskmelon C*ucumis Melo* L. (Cucurbitaceae) in the Pest Grasshopper *Zonocerus Variegatus* L. (Pyrgomorphidae)

By Ngnaniyyi Abdoul, Seino Richard Akwanjoh & Dongmo Ingrid Tonleu

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Abstract- The Muskmelon, *Cucumis melo* L., is a Cucurbitaceae widely cultivated in Cameroon for its nutritional and ethnomedicinal benefits. Species of Cucurbitaceae are known to contain several bioactive molecules that include the terpenoid cucurbitacins, which has been shown to cause significant molting defects and mortality in a variety of Coleoptera insect species such as *Leperesinus fraxini* PANZ(Coleoptera, Scolytidae) *Stereonychus* fraxini DE GEER (Coleoptera, Curculionidae). This study was designed to determine if an aqueous extract of Muskmelon, *C. melo* var. *Cantaloupensis Americana*, could profoundly affect the meiotic process in the Orthoptera grasshopper *Zonocerus variegatus,* a veritable food crop pest in Africa south of the Sahara. Different concentrations (0 µg/ml, 5µg/ml, 10 µg/ml, 20 µg/ml, 30 µg/ml, and 40 µg/ml) of aqueous extract of the leaves, fruits, and roots of *C. melo* were, respectively injected using the intraperitoneal method (into the hemocoel) of new reproductive and adult male individuals of *Z. variegatus*. Cytogenetic analysis revealed that Muskmelon extracts significantly reduced meiotic indexinduced meiotic chromosome abnormalities and significantly reduced chiasma frequency.

Keywords: cytotoxicity, genotoxicity, cucumis melo L., aqueous extracts, meiotic process, meiotic index, chromosomal abnormalities, zonocerus variegatus L.

GJSFR-G Classification: DDC Code: 583.46 LCC Code: SB351.C8



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Keywords: cytotoxicity, genotoxicity, cucumis melo L., aqueous extracts, meiotic process, meiotic index, chromosomal abnormalities, zonocerus variegatus L.

I. Introduction

The Muskmelon, *Cucumis melo* L. (Cucurbitaceae), is variously known in Cameroon as the melon. In addition to *Citrulus lanatus* (Thunb.) (water melon), *C. melo* is one of the important cultivated cucurbits in Cameroon. *C. melo* L. is an annual creeping plant with long stems tendrils, large rounded heart-shaped green leaves, as well as large and round fruits that may be embroidered with white spots [1,2]. The muskmelon is cultivated especially in the North West, and West Regions where whole fruits and dried seeds are commonly sold on the Cameroonian markets [3]. The mesocarp of the fruitis eaten, the seeds used as a thickener in many Cameroonian soups. The species is an important and valuable vegetable crop in Cameroon and several tropical countries. It is widely consumed for its nutritional value and used for a wide variety of traditional medicinal properties [4-6].The family Cucurbitaceae is an economically significant group of plants that contains bioactive phytochemicals such as Glycosides, Terpenoids, Saponins, Tannins, Steroids, and Carotenoids [7]. The terpenoids contain the bitter-tasting bioactive principle, cucurbitacins, compounds that have curative and several biological activities [3,8-15]. Cucurbitacins are essential for their therapeutic use in cancer treatment and other ethnomedicinal activities. They have also been linked with controlling several beetle pests of Cucurbitaceae plants. Cucurbitacins are very effective in natural plant defense against herbivores [16]. Cucurbitacin B, a variety of this principle, has been shown to significantly reduce the adult longevity and fecundity in the melon aphid, *Aphis gossypii*[17,15]. It has also been shown to have potent antifeeding properties for insects not adapted to exploiting cucurbits. Four beetles, *Popillia japonica* Newman, *Ceratoma trifurcata* (Forster), *Leptinotarsadecemlineata* (Say), and *Trichoplusia ni* (Hubner), were reported to stop feeding on application of cucurbitacin B to appropriate sources [18]. Cucurbitacin has been shown to affect oviposition in the moths *Ostrinianubilalis* (Hubner), and *Spodopteraexigua* (Hubner) females [18]. Available literature indicates that the bio-pesticidal activities of cucurbitacin have been extensively investigated for beetles of the Coleoptera order. On the other hand, information on the pesticidal effects of cucurbitacin on other pest species, especially the Orthoptera grasshoppers, a vital pest group in Cameroon and Africa, is not available in the literature. *Zonocerus variegatus* L., has been variously shown to

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be a veritable pest of both food and cash crops in Cameroon and several Central, East, and West African countries. This grasshopper is a severe problem because of its wide host ranges, and its economic and ecological cost. Thus far, this grasshopper pest has been controlled solely with chemical pesticides such as Malathion. Therefore, the search for effective bioactive substances in the control of the pest continues. The present study was designed to determine the effects of the bioactive compounds in *C. melo* on the meiotic process in the pest grasshopper *Z. variegatus* L. It is expected that the extract will affect the meiotic process in the grasshopper which lead to the disruption of the reproduction process in this grasshopper pest species and hence valuable to controlling the pest population.

II. Materials and Methods

a) Raw Materials and Extraction

The leaves, fruits, and roots of *C. melo* used for this study were collected from a farm in Balessing, a village in the Menoua Division of the West Region in Cameroon. The farm was located at latitude 5°30'2"N and longitude 10°14'39"E. It was free of the use of fertilizers and pesticides. Leaves, ripe fruits and roots were collected only from mature plants. The species and variety which was easily recognizable were authenticated by Dr. NGANSOP Eric of the National Herbarium in Yaoundé with reference N080613/SRF/Cam of 20/04/2022. The plant materials collected were taken to the laboratory, and washed of dust and ground before extraction. The different plant parts were individually chopped into small bits to accelerate drying and then dried in an oven at 60°C until there was no weight change. These specimens were next ground into powder. To prepare the aqueous extracts, 100 g of each sample was individually macerated in distilled water and stored at room temperature while constantly stirring at regular intervals with a spatula. After 36 h, the mixtures were filtered using a sieve of 150 µm in diameter and then with a coffee filter paper no. 4. The filtrates obtained were treated to 60°C in an oven to obtain whitish powders with total dry weights of 34.18%, 34.69% and 33.56% for leaves, ripe fruits, and roots, respectively. These were used to prepare stock solutions for leaves, ripe fruits and roots, respectively. From the stock solutions, micro dilutions of 5 µg /ml, 10 µg /ml, 20 µg /ml, 30 µg /ml, and 40 µg /ml were prepared by the addition of distilled water.

b) Experimental Animals

Eighty (80) adult males of *Zonocerus variegatus* collected on campus in the University of Dschang (West Region of Cameroon) were brought to the Laboratory of the Research Unit of Biology and Applied Ecology (RUBEA) of the University of Dschang. Before the animal studies, the grasshoppers were reared in mineral water bottle cages and fed with fresh leaves (4 g per day per individual) of bitter leaves (*Vernonia amygdalina*), two days.

c) Administration of Extract

The grasshoppers were divided into groups of five individuals and labeled I, II, III, IV, V & IV. The grasshoppers in groups I, II, III, IV, & V were respectively used for the evaluation of the aqueous extracts of leaves, fruit, and roots of *C. melo,* while those in group IV were used as the control. Each of these groups was further divided into five subgroups groups of A, B, C, D & E, each containing five (05) insects. Grasshoppers in groups A, B, C, D & E were respectively injected peritoneally (in hemocoel) with 0.1ml of 5 µg/ml, 10 µg/ml, 20 µg/ml, 30 µg/ml, and 40 µg/ml of aqueous extract. The treated grasshoppers were allowed an incubation period of 96 h before being anesthetized and dissected for the testes. Grasshoppers in group F were the control.

d) Preparation and Analysis of Chromosome Smears

The chromosome smears were prepared using the method of [19], and the smears were examined with the help of 10X, and the 40X objectives of the Fisher binocular compound light microscope. The meiotic smears obtained were examined for abnormalities that included laggards, bridges, sticky chromosomes, vagrant chromosomes, and breakages.

e) Meiotic Index

The slides prepared for the extracts were observed under the microscope to record the Number of non-dividing and dividing cells. The Meiotic index was calculated using the formula[20]:

$$\text{Meiotic Index (\%)} = \frac{\text{Number of dividing cells recorded}}{\text{Total number of cells examined}} \text{X } 100$$

f) Photographs

Photographs of chromosome aberrations present were made using a Techno, Camon 16 phone mounted with a 48M AIQUAD Camera.

g) Statistical Analysis of Data Collected

The Python 3.1 statistical software Pandas package was used for this analysis. The mean of the different types of chromosomal abnormalities as well as the other in-depth meiotic parameters, were subjected to the one-way ANOVA test followed by the Tukey posthoc test (HSD) at the level of significance of $p<0.05$ [21].

III. Results and Discussion

a) Cytogenetic Analysis

After staining testicular follicles of *Z. variegatus* treated with aqueous extracts of *C. melo*, the Orcein-stained cells were analyzed with the 10X and 40X objectives of the compound light microscope. The results on the meiotic index, meiotic behavior of

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chromosomes, and chiasma frequency obtained are discussed in this section.

b) Meiotic Index

Fig. 1 revealed that the meiotic index decreased with increase in the concentration of extract. The meiotic index for treatments with the leaves of *C. melo* was not much compared to that observed for fruits and roots. A significant decrease in the meiotic index was recorded for the highest concentration (40 µg /ml) of extract of roots.

Table 1: Effects of aqueous extracts of leaves, fruits, and roots of *C. melo* on the meiotic index of the cells of *Z. variegatus*

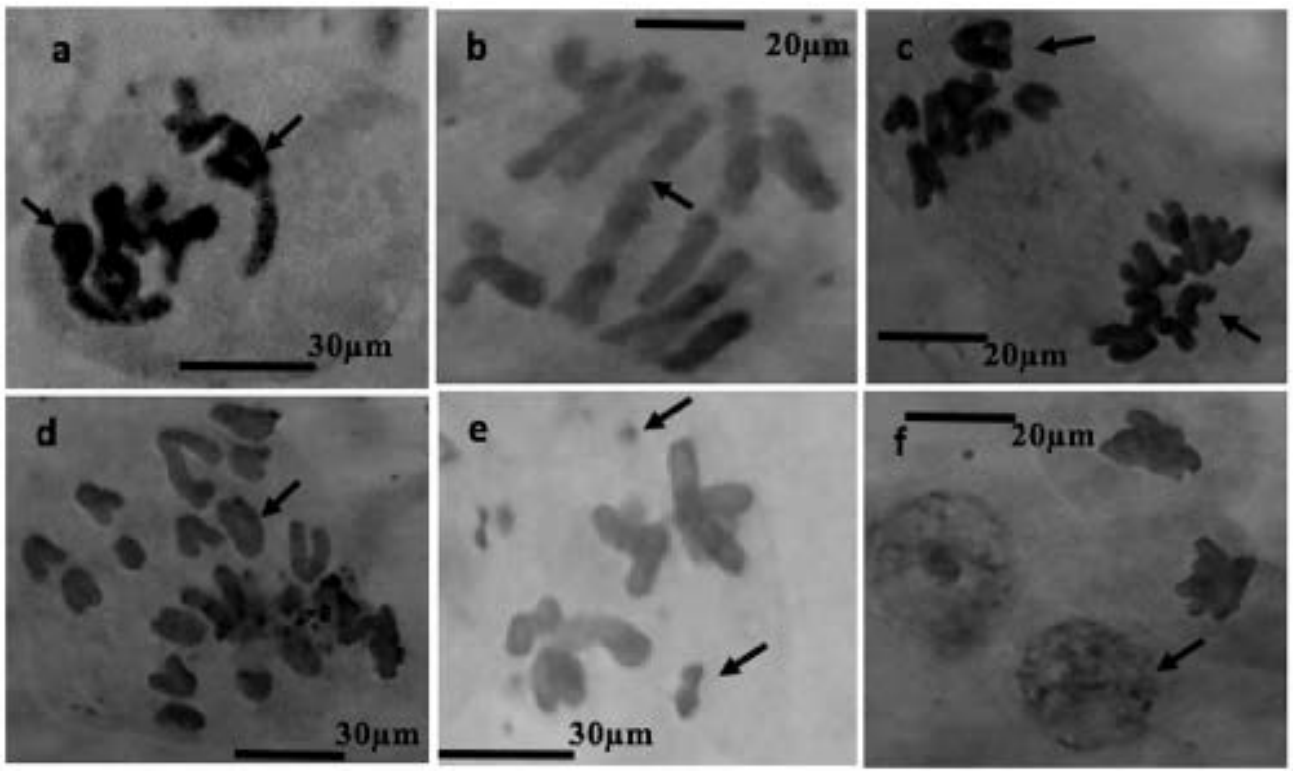
Treatments	Meiotic Index (MI) 100%
Tape water (Control)	14.25 ± 4.69^{a}
Leaves	9.45 ± 3.45^{c}
Fruits	8.80 ± 3.28^{b}
Roots	8.00 ± 2.2^{b}

Values are means ± SEM. A number of trials n=5. Groups that have no letters in common differ significantly different from the control group (Distilled water), applying one-way ANOVA followed by Tukey's post-test (HSD)

The mean meiotic indices obtained for leaves, fruits, and roots of *C. melo* (Table 1) were subjected to ANOVA. This analysis revealed that the mean meiotic indices obtained were significantly lower than the control. However, the meiotic indices induced by the extracts of fruits and roots were not different. The antimeiotic activity of the extracts can be linked to the distribution of cucurbitacins (the bioactive principle) in the leaves, fruits, and roots *C. melo*. Cucurbitacins have been reported to be more concentrated in the stem and roots of Cucurbitaceae plants than in other parts of the plant [2], and in the fruits [22]. These results indicated that the aqueous extracts from the leaves, fruits, and roots of *C. melo*at certain concentrations, have antimeiotic properties.

c) Meiotic Behaviour of Chromosomes

With the objective to investigate the effect of the extracts on the meiotic behaviour of the chromosomes in *Z. variegatus*, the chromosome smears prepared were examined for abnormal behaviour. This was important because the behavior of the chromosomes would determine whether treatment with the extracts would produce normal or abnormal spermatozoa and hence affect the meiotic reproductive. An analysis of the smears revealed various degrees of abnormalities depending more or less on concentrations of the extract applied. Several types of chromosome abnormalities that included sticky Metaphase 1, bridges, and lagging chromosomes in Anaphase 1, and chromosome fragments were recorded (Fig. 2a - e).



(a= Sticky chromosomes; b= Anaphase I with bridge; c= laggard arrowed; d= laggard arrowed; e= Chromosomes break; f= Ghost cell)

Figure 2: Various chromosome deformities in *Z. variegatus* on treatment with aqueous extracts of *C. melo*

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It is essential to mention here that sticky chromosomes and bridges could result in chromosome breakages in the course of meiosis and hence produce acentric fragments. Acentric chromosome fragments lack centromeres that are essential for the division and the retention of the chromosome in the cell. Acentric chromosomes fragments are therefore lost when the cell divides. The loss leads to unbalanced gametes and could result in infertility. Lagging chromosomes contribute to the uneven distribution of chromosomes and, therefore,to the formation of cells with the abnormal numbers of chromosomes. These abnormalities, called aneuploidy, could be amongst the major causes of infertility in animals and plants [23-25] and could be used in the biocontrol of pest grasshoppers. Many translucent cells were observed in various frequencies during this study. The outline of the cells that occurred singly was visible, but the nucleus and cytoplasmic structures were not stainable (Fig. 2f). As per the definition of [26] such cells could be described as ghost, shadows or translucent cells. They have been variously recorded in human samples and associated with cancers. They are often swollen or enlarged cells that do not have nuclei [26]. Records of ghost cells in grasshopper species were not available in cytogenetic literature. Therefore, this report is a pioneer record for ghost cells in Orthoptera. There is no knowledge about their origin, nature, significance and relation to meiosis. During this study, the frequency of ghost cells was observed to be concentration dependent. Hence it is suggested that their presence is an indication of high-level cytotoxicity of the extract. In conclusion, meiotic abnormalities lead to morphological and genetic variations, which bring about not only evolution but also intraspecific reproductive barriers. Such reproductive barriers could be exploited for pest control.

d) Chiasma Frequency

The importance of chiasmata in a population cannot be overemphasized. Individuals with high chiasma frequencies are considered robust, while those with low chiasma frequencies are unstable and can be easily affected by the environmental changes. Therefore, small changes in climate affect populations with low chiasma frequencies, and can lead to, drastic reduction in population size. Chiasma formation in control was normal. The individuals had mean chiasma frequencies of 11.90, which is normal and was not at variance with the observations of [27]. On the other hand, chiasma frequencies in individuals in the treatments experienced significant reductions (Fig. 3); Chiasma frequency was, therefore, inversely proportional to the concentration of extract. These observations are at variance with the report of [28], who recorded an increase in mean chiasma frequency in individuals of *Z. variegatus* treated with aqueous extracts of *Annona muricata*. The present data could not explain this difference.

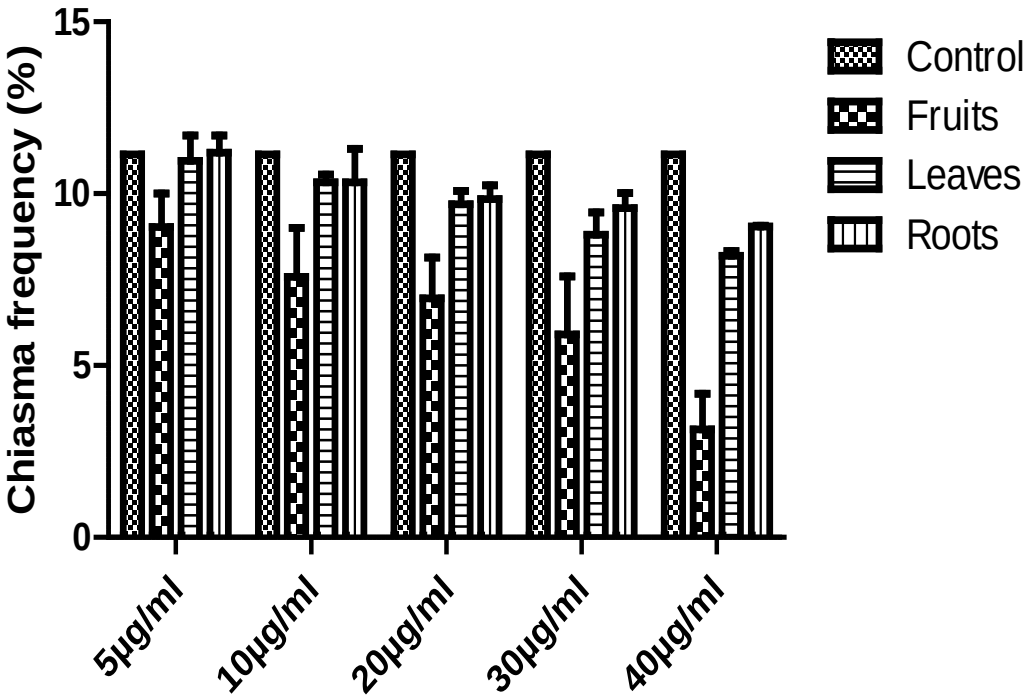

Figure 3: Chiasma frequency in *Z. Variegatus* treated witch extracts of *C. melo*

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Table 2: Effect of aqueous extracts of leaves, fruits, and roots of *C. melo* on chiasma frequency of the cells of *Z. variegatus*

Treatments	Chiasma frequency (%)	Mean percent chiasma frequency per bivalent
Tape water (Control)	11.90±1.70[a]	1.32±0.168[a]
Leaves	10.01±0.3[b]	1.11±0.28[a]
Fruits	8.20±1.19[d]	0.91±0.88[c]
Roots	9.11±0.86[c]	1.01±0.73[b]

Values are means ± SEM. The number of trials n=5. Groups that have no letters in common differ significantly different from the control group (Distilled water), applying one-way ANOVA followed by Tukey's post-test(HSD)

Analysis of the mean chiasma frequencies recorded (Table 2) revealed that the different extracts induced the formation of chiasmata differently, with the extract of fruits causing the lowest mean chiasma frequency. In all the treatments, induction was significantly lower than for the control. It is worth noting that chiasmata areessential for the attachment of homologous chromosomes in bivalents and hence subsequent segregation to the poles at Anaphase 1. Therefore, chiasmata are crucial for producing normal and genetically balanced spermatozoa and hence reproductive success in a population [29]. Available evidence shows that populations with decreased chiasma frequencies are less stable and are unable to adequately withstand sudden changes in the environment as compared to the populations with increased chiasma frequencies that can adapt to sudden changes in the environment [30,31]. The effect that decreased chiasma frequency in this study was difficult to assess from these results.

IV. Conclusion

The aqueous extracts of the Muskmelon, *C. melo* can induce cytogenotoxic changes in the meiotic cells of the pest grasshopper *Zonocerus variegatus*,and hence affect fertility. At high concentrations of the aqueous extract, cytogenotoxic changes induced could be drastic and a reduction lead to reduction in future populations. Therefore, at sufficiently high concentrations, extracts of *C. melo* could be used in the formulation of biopesticides to control the pest grasshopper *Z. variegatus*. The following research will test these extracts on a few ovarian and testis cancers induced in Wistar rats.

Abbreviations list

Not used

Declarations

Ethical approval and consent to participate

All experimental studies on plants and grasshoppers have complied with relevant institutional, national and international guidelines and legislation.

Consent to publication

We declare hereby that this work has not been published or accepted, in whole or in part, and that it is not selected for publication in another journal. All authors have approved the manuscript and agree with its submission in Global Journal of Science Frontier Research: GBio-Tech and Genetics.

Availability of data and material

Datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

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Author Contributions

- N.A. and S.R.A. conceived and conducted research experiments,
- N.A. and D.T.I. analysed data and conducted statistical analyses,
- N.A. and S.R.A wrote the manuscript,
- S.R.A. reviewed the manuscript. All authors read and approved the manuscript.

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Cross - Century Discovery: Mendelian Dualistic Genetics

By Muying Zhou

Abstract- This article is a summary and review of new insights into Mendel's gene assumption since its rediscovery in 2018. This is a cross-century discovery. The gene assumption told the world that the gene is the element that makes individuals being with parental specifications. The gene is the facilitator rather than the producer. New individuals with parental specifications are produced by the facilitator and its recipient in cooperation. This is conceptually roughly the same as making airplanes with two elements, the drawing and the production line. Thus, the hereditary material should consist of two elements; genetics is dualistic. T.H. Morgan, who founded the theory of the gene and regarded the gene as the producer of individuals, completely misunderstood Mendel, and pulled Mendelian genetics back into the mire of monism. O.T. Avery *et al.* confirmed that genes are DNA, thereby ushering in the era of the physical verification of the truth of genes. The scientific facts gathered in later 60 years finally prove that any individual, animal or plant (including single-celled organisms), is the product of the transcription of its DNA (genome) by the transcriptase (system) of an egg. In other words, the hereditary material able to give rise to the individuals consists of egg transcriptase (system) and DNA (genome).

Keywords: *genes, hereditary material, genetics, template, transcriptase.*

GJSFR-G Classification: *UDC code: 575.112.2*



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Keywords: genes, hereditary material, genetics, template, transcriptase.

I. Introduction

The hereditary material able to give rise to the individuals is the basis of genetics. So "What produces the individual" became the first question of genetics. Before the biological matter was clarified, people can only put forward hypothetical answers such as "miniature", "gemmules" and "Germ-plasm" using their imaginations. Each hypothetical element of these answers had two abilities: the ability to produce and the ability to make the individual meeting parental specifications. This is the inherent feature of monistic genetics. Without either of the abilities, the hereditary material is not able to produce individuals with parental specifications. The modern theory of heredity is rooted in genes discovered by Mendel and influenced by habitual thinking of the monistic genetics, resulting in people of the early last century conveniently regarding genes as the hereditary material. However, whether such an understanding is correct or not can be answered by whether genes have the above two inherent abilities. In 1944, genes were confirmed as DNA; thus, the answer was coming. Finally, science proved that genes (DNA) are only the template controlling individual specifications and have no producing ability. Gene-monistic genetics comes in a hopeless situation. Facing a hopeless situation is undoubtedly very painful, but it can also be the eve of a new breakthrough. When we went back to Morgan's <The theory of the gene> [1], we found the Mendel's gene assumption, and the gene it defined was completely consistent with the fact of today's DNA template: The gene is only an element that makes individuals being with parental specifications. Thus, Mendelian genetics is actually dualistic genetics.

II. The Hereditary Material and its Identification Standards

The hereditary material refers to the material transferred from the parent to the offspring (in the fertilized egg) that is responsible for biological hereditary facts. However, this only indicates from where the hereditary material originates and its logical responsibilities. However, there is no explanation or clarification for why it can, or should, be responsible for the hereditary facts.

Heredity is a well-known fact. It refers to parent–child traits being similar, or even the same, in living things. On the basis of data, we can conclude that the hereditary material is the producer of individuals, and it usually exists completely in the fertilized egg. These data are: 1) The fertilized egg is the direct and only material source from the parent to the offspring; 2) Eggs (fertilized) can produce chickens (individuals); 3) The producer should be responsible for everything in the product (it is natural. Just as if your TV set is broken, then who is responsible? The producer. Who is responsible for an aircraft crash due to a defect in the aircraft itself? The aircraft producer of course).

Thus, we realize that the hereditary material can, or should, be responsible for the hereditary features only because it is the producer of the individual. This leads us to the first gold standard for identifying the hereditary material: *the hereditary material must be the producer of the individual.* Surprisingly, the first gold standard naturally leads us to the second gold standard for identifying the hereditary material: *it must be self-replicating.* It is linked with reproduction. We can prove it

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with the following: 1 cell → 2 cells, where "→" represents producing. Thus, the formula states that the first cell produced a new cell. The cells here could be single-celled organisms, like bacteria. Who is the producer? Of course, it is the hereditary material. Every cell contains the hereditary material, so we can write cell as "cell (the hereditary material)". Consequently, the formula above becomes the following: 1 cell (the hereditary material) → 2 cells (the hereditary material). Note: The pre-formula hereditary material produces a new cell, and the new cell produced in the formula also contains the hereditary material. That is to say, the hereditary material has produced a copy of itself when it produces a new individual. Thus, the hereditary material is self-replicating material.

III. Monistic Genetics and Dualistic Genetics

The theory claiming that the hereditary material should contain only one element belongs to monistic genetics, and the theory claiming that the hereditary material should contain two elements belongs to dualistic genetics.

a) Pre-Mendelian Genetics: Preformation, Pangenesis and Germ-plasm theories

Preformation, Pangenesis and Germ-plasm theories assumed "miniature", "gemmules" and "germ-plasm" respectively, as the hereditary material. Each of these theories belongs to monistic genetics. As the hereditary material of monistic genetics, "miniature", "gemmules" and "germ-plasm" each should have two capabilities: that of production and that of making individuals meeting parental specifications. Unluckily, such wonderful material has not yet been found in the real world. So, these theories have not yet been supported by any scientific facts.

b) Mendelian genetics

Mendel did not aspire to create a new theory of heredity, and he did not offer any answers to the first question. He submitted a paper on a plant hybridization experiment. He described a very specifically designed experiment that posed a new question that was completely different from the "first" question above and opened a new door on genetics.

i. *Mendel's experiment and its results*

Mendel used pea plants of different specifications (varieties; he listed at least seven pairs of different specifications, including individual height, seed color, seed smoothness and flower position) for hybridization experiments. A typical experiment was as follows: "He crossed a tall variety of edible pea to a short variety. The offspring, or hybrids, F_1, were all tall. These were allowed to self-fertilize. Their offspring were tall and short in the ratio of three tall to one short" [1].

ii. *The experimental result raised the second basic question of genetics*

The question raised by such experiments is "Why are there always two types of offspring from a cross between tall and short varieties (pea): one being the tall plants with the same specification as the tall variety, and the other being the short plants with the same specification as the short variety? Even when the F_1 were all tall plants, after F_1 self-fertilization, the short plants appeared again in the offspring". This question does not ask "What produces the individual"? at all, so it is not the "first" question of genetics. We refer to it as the second basic question of genetics [2]. This question is similar to "Why do our plant produced some planes that were big and some that were small? Why were the first batch all big? Why were the last batch big and small in a ratio of 3 big to 1 small"? It asks "What controls the specifications of the product"? We know that the specifications of the aircraft are controlled by the blueprints. Following the specifications implicit in the big aircraft blueprints we produce big aircrafts, and following the specifications implicit in the small aircraft blueprints we produce small aircrafts. Therefore, the questions raised by the experiment inspire us to assume whether the parents of tall and short varieties have their own hereditary elements controlling the product (individual) specifications, whether following the tall specification version can produce tall plants, and following the short specification version can produce short plants? The "second" question usually arises in sexually reproducing species (or crosses between two varieties). Because there are two parents (father and mother), for the producer (the fertilized egg), a new question arises: which version to choose between the father and mother versions to produce individuals? In daily life, the "second question" leads people to ask questions such as "Why is Tom's oldest son tall like Tom, while the younger son is short like Tom's wife? " "Why is Tom's son's nose similar to Tom's nose, but the ears are similar to those of Tom's wife? " or "Why do the noses of the Habsburg family seem to be produced from only one template"? etc.

iii. *Mendel's gene assumption and his dualistic genetics*

In response to the above question, Mendel proposed the gene assumption: "if the tall variety contains in its germ-cells something that makes the plants tall, and if the short variety carries something in its germ-cells that makes the plants short"[1] (Here the "something" is what was later called a "gene"). Mendel then assumed that the tall gene is dominant and the short gene is recessive, which successfully explained the phenomenon that F_1 all tall, and F_2 tall and short in the ratio of three tall to one short.

"The gene can make the individual (plant) to be tall or short (specification)", so it is the facilitator making the individual to be parental (tall or short) specification

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rather than the producer of the character. One specification is controlled by a gene (or a group of genes), and seven specifications listed by Mendel would be controlled by seven genes (or seven groups of genes); Therefore, all specifications of an individual should be controlled by a set of genes (genome).

Being only a facilitator, the gene cannot produce individuals alone, only by collaborating with the receptor that accepts facilitation can the gene achieve the facilitation result. For example, a politician promises the voters that "I can make this country great again". Here, he does not mean he can achieve this goal alone. As the facilitator, he can complete it only in cooperation with the people (as recipients) who accepts his path. So, the implicit logic of the gene assumption is that the hereditary material is composed of two elements: one is the gene controlling the individual specifications, and the other is the producing element that accepts the gene limit to perform the operation. The individual is produced by the producing element in the fertilized egg following the specifications limited by the gene (genome). Thus, Mendelian genetics is dualistic genetics [2,3,4.5].

iv. *The support of scientific facts for Mendelian dualistic genetics*

In 1944, Avery *et al*. confirmed that genes are made of DNA, and stated: "DNA is capable of stimulating unencapsulated R variants of *Pneumococcus* Type II to produce a capsular polysaccharide" [6]. This proved that "DNA can make *Pneumococcus* to be S-type (encapsulated) specification". Contrast this with "Genes can make the individual (plants) to be tall or short (specification)", The two sentences' pattern are exactly the same, but the corresponding words are different: genes are replaced with DNA, plants are replaced by *Pneumococcus*, and tall or short specifications are replaced by S-type (encapsulated) specification. It is indeed a miracle that Mendel's gene assumption has been confirmed by such consistent experimental facts 79 years after it was proposed (especially in the era when genes were misunderstood as the dominant ideology of individual producers).

After 1944, the substances that produced the individual in the fertilized egg were quickly identified. When the fertilized egg initiates egg's transcription, the activity of producing the individual begins. In transcription, DNA is the template, and as the template DNA completely matches the gene defined by Mendel; The only material that performs producing operation on the template is egg's transcriptase(s), ·so it is the producing element. There is no possibility of identifying other substances as producing element. That is to say, the two elements underlying the Mendel's gene assumption that form the hereditary material can be affirmed. Nevertheless, the authenticity of these two elements needs to be verified using the gold standards for identifying the hereditary material in the next section. Only those that meet the gold standards are truly the hereditary material.

c) *The theory of the gene, so-called modern theory of heredity (The bad result of ignoring Mendel's gene assumption -- Mendelian dualistic genetics is misunderstood as monistic genetics)*

Finally, we have to discuss the theory of the gene, so-called modern theory of heredity. It claims to be Mendelian genetics, but in fact, it is monistic genetics that arose from misunderstanding Mendel. Mendel's gene assumption cited in this paper from Morgan's <The theory of the Gene>. But there is no mention in the book that Mendel ever proposed the gene assumption. This clearly indicates that Morgan hardly noticed Mendel's definition of genes as the facilitator. But this did not delay his conclusion that "the characters of the individual are referable to genes" [7] and "So long as a complete set of units (genome) is present, the power to produce a new whole is potentially given"[8]. That is to say, Morgan saw Mendel's proposal of "something (gene)", but did not put in effort to distinguish between facilitator or producer. Because he believes that the gene is no different from the "miniature" "gemmules" and "germ-plasm" in history, that is, the hereditary material able to give rise to the individuals. After genes are confirmed as DNA, we can use the gold standards for identifying the hereditary material to test the correctness of the theory of the gene. Molecular biology tells us that DNA is a template, DNA has no producing capacity, DNA can't build 3', 5'-phosphodiester bonds. Then the conclusion is certain: 1) Due to the lack of producing ability, DNA cannot be the producer of the individual. 2) Because DNA cannot establish 3 ', 5' - phosphodiester bonds, DNA cannot replicate itself (DNA can only rely on DNA replicase to obtain passive replication). Visible genes do not meet the two gold standards for identifying the hereditary material. The theory of the gene, so-called modern theory of heredity is a failed theory.

IV. Determining Whether Transc × DNA is the Hereditary Material

We can use Transc × DNA to represent the union of the egg's transcriptase and genome (DNA). Now, we need to prove that Transc × DNA meets the two gold standards for identifying the hereditary material.

a) *Transc × DNA can produce the individuals*

The life of the fertilized egg is launched by Transc × DNA, Transc × DNA creates egg transcription. The fact is that any individual, whether animal or plant, is the product of a natural, preprogrammed, causally continuous and autonomously

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producing process caused by the transcription of the genome (DNA) by the transcriptase(s) of the fertilized egg (unicellular organism included). Without egg transcription, no new individuals (new living things) could come into being. These are all undeniable objective facts. Not only does the new individual result from egg transcription, but also an individual in any phase of life results from this producing process (the individual in A, B..... Z phase is the result of this process progressing to the A, B..... Z phase, respectively, such that the individual in the N phase is the result of this process progressing to the N phase). An embryo is the result of this process progressing to the embryonic stage, a juvenile individual is the result of this process progressing to the juvenile stage, and an elderly individual is the result of this process progressing to the declining stage. Death is the result of the termination of this process owing to internal or external causes. It cannot be denied that an individual's life is a continuous process. During this process, the individual existing in each second is the automatic result of the individual existing in the previous second. Once this producing process is started, only death can terminate it. To put it another way, once the automatic and continuous producing activity initiated by egg transcription stops and no longer resumes, the individual's life is terminated. Thus, Transc × DNA not only can produce individuals, but is also the source creating an individual's life.

The fact that the egg's transcription leads to the life of the individual can be seen throughout the world. In a chicken farm, after thousands of eggs enter the incubation room and egg transcription is initiated, the emergence of thousands of chickens is a predictable fact. Even the whole life course of these chickens before being slaughtered has an inherent causal relationship with egg transcription. In silkworm-rearing workshops, when tens of thousands of fertilized eggs start transcription, the appearance of tens of thousands of young silkworms is expected with a high probability, and death is the only accident that can stop the activities led by an egg's transcription. The eons of survival history of various single-celled organisms are also the result of egg transcription in the first cells of these species. Transc × DNA is the root substance responsible for all these facts.

A fact that must be stated: In this section you can see that egg transcription initiates a continuous automated producing process, much like the automatic producing process of the car. An automatic automotive production line contains a series of accessories to ensure the appropriate and reasonable output of various components in time and space smoothly throughout the assembly process. Similarly, the transcriptase operating on the template also requires a series of transcription factors as attachments to ensure the appropriate and reasonable output of various products in time and space smoothly throughout the assembly process. Thus, to be precise, the producing element is a series led by the egg transcriptase that contains the complete set of transcription factors prestored in the egg, *the egg transcriptase system.* However, there is no doubt that the transcriptase is the leader of this system, and it is the only factor that actually consumes energy, does work and is responsible for establishing 3', 5'-phosphodiester bonds [3,5].

b) Transc × DNA can produce itself

Because Transc × DNA is the maker of the individual, it must be able to replicate itself owing to their inevitable connection. In the aforementioned formula: 1 cell (the hereditary material) → 2 cells (the hereditary material), if we change (the hereditary material) to (Transc × DNA), then we get the following: 1 cell (Transc × DNA) → 2 cells (Transc × DNA). The Transc × DNA in the first cell is the producer of the new cells, but the new cells also contain Transc × DNA. Thus, the first Transc × DNA has produced a new Transc × DNA, showing that Transc × DNA can replicate itself.

V. Conclusion

The Mendel's gene assumption leads us to find Mendelian dualistic genetics, the ultimate scientific genetics. Scientific facts have shown that the hereditary material able to give rise to the individuals consists of egg transcriptase (system) and DNA (genome). Transc × DNA not only is the source of producing the individuals, but also is the source of the individuals' lifelong existence.

Acknowledgments

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[For Peer Reviewer]

Highlights of this manuscript

1. *Correctly realize the Mendel's gene assumption*: "if the tall variety contains in its germ-cells something that makes the plants tall, and if the short variety carries something in its germ-cells that makes the plants short"[1] (Here the "something" is what was later called a "gene"). "The gene can make the individual (plant) to be tall or short (specification)", so *it is the facilitator making the individual to be parental (tall or short) specification rather than the producer of the character.* Being only a facilitator, the gene cannot produce individuals alone, only by collaborating with the receptor that accepts facilitation can the gene achieve the facilitation result. *For example, a politician promises the voters that "I can make this country great again".* Here, he does not mean he can achieve this goal alone. As the facilitator, he can complete it only in cooperation with the people (as recipients) who accepts his path. So, the implicit logic of the gene assumption is that the hereditary material is composed of two elements: one is the gene controlling the individual specifications, and the other is the producing element that accepts the gene limit to perform the operation. The individual is produced by the producing element in the fertilized egg following the specifications limited by the gene (genome). Thus, Mendelian genetics is dualistic genetics.
2. The gold standards for identifying the hereditary material
   - The hereditary material must be the producer of the individual
   - The hereditary material must be self-replicating.
3. Compare the following two sentences:
   - 1865 Mendel said that genes can make the individual (plants) to be tall or short (specification);
   - 1944 Avery *et al*. said that DNA can make *Pneumococcus* to be S-type (encapsulated) specification".

It is indeed a miracle that Mendel's gene assumption has been confirmed by such consistent experimental facts 79 years after it was proposed (especially in the era when genes were misunderstood as the dominant ideology of individual producers).

4. *New discovery:* Transc × DNA (represent the union of the egg's transcriptase and genome) not only is the source of producing the individuals, but also is the source of the individuals' lifelong existence.

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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 Science Frontier Research Paper

Techniques for writing a good quality Science Frontier 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 science frontier 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.



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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:

- Explain the value (significance) of the study.
- 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.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- 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:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- 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:

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



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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:

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

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- 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.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- 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.
- 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?
- 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

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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

INDEX

A

Adipogenic · 28, 33
Alopecia · 13
Anomalies · 17
Apoptosis · 20, 22, 30, 93

C

Causative · 88
Chamomile · 13

E

Endothelium · 1, 2

F

Fibrillation · 4, 14

G

Gladiolus · 59
Glioma · 20

I

Idiopathic · 88, 95, 96

L

Licorice · 13

M

Metastasizes · 24

N

Necrosis · 5, 6, 13, 93

O

Oligoarthritis · 88, 89, 92

S

Scoliosis · 5
Sporadic · 19, 34
Synovial · 23, 26, 27, 28, 35, 93





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