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A comparison of salivary Indian hedgehog (IHH) protein levels and cervical maturational stages as growth indicators

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Abstract
The study was done to assess the salivary Indian hedgehog protein levels in the pre pubertal, pubertal and post pubertal orthodontic patients and to correlate the levels to CVMI stages. The samples consisted of 90 patients aged 08-20 years (42 males, 48 females), grouped into 6 cervical stage according to their cervical maturation. 3ml of saliva was collected and ELISA was done. The results suggested that there was a significant rise in the mean salivary Indian hedgehog levels from pre-pubertal to pubertal phase and a gradual decline in the levels from the pubertal to post pubertal phase. There was statistically significant result (P=0.03) for the comparison of mean salivary Indian hedgehog levels between different Cervical Vertebral Maturation stages and there was no significant difference between the gender. Thus, Salivary Indian hedgehog can be used as bio-marker to determine the skeletal maturity of an individual.

Keywords: Skelatal maturity, CVMI stages, saliva, Indian hedgehog, growth

1. Introduction
Skeletal maturation is an integral part of an individual's pattern of growth and development. Accurate determination of skeletal maturity and remaining growth is crucial for many orthodontic, orthognathic and dental implant decisions. The chronologic timing of puberty and the adolescent growth spurt demonstrate much variation and are affected by both genetic and environmental factors [1].

The degree of skeletal development is a reflection of the level of physiological maturation of a subject. Bone age was shown to be as important as chronological age in evaluating an adolescent's physical development. The determination of skeletal age indicates how much further growth, a child will attain and allows the prediction of final height [2].

Certain skeletal developmental stages of the hand and wrist have been shown to be closely associated with the pubertal growth spurt and these radiographs have been used as an indirect method for the assessment of somatic maturity stage. However, the routine use of hand wrist radiographs has lately been questioned from the radiation hygiene, safety point of view [3].

Cervical vertebral stages and hand-wrist radiographs are currently used to identify the peak mandibular bone growth. These are highly subjective techniques that not only involve radiographic exposure but also lack the ability to determine the intensity of the growth spurt and the end of growth [1].

The Important Blood Panel biomarkers for growth hormone production are Insulin like Growth Factor- I (IGF-1), sex hormone, Osteocalcin, BP3/BP2 Ratios (IGF Binding Proteins), Dehydroeplandrosterone (DHEA), Thyroid Stimulating Hormone (TSH), Pregnenolone, and Thyroid free T3, Thyroid free T4, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) [4].

Various systemic and local factors regulate craniofacial growth. Local factors involved in regulation of chondrocyte activity and subsequent endochondral bone growth include Indian hedgehog protein (Ihh), parathyroid hormone related protein (PTHrP), FGF, BMPs, VEGF, SOX 5, 6, 9, RANKL, and OPG [2]. Indian hedgehog protein has been reported to regulate multiple steps in the development of growth plate and the secondary cartilage at the head of the condyle during skeletal morphogenesis [6].

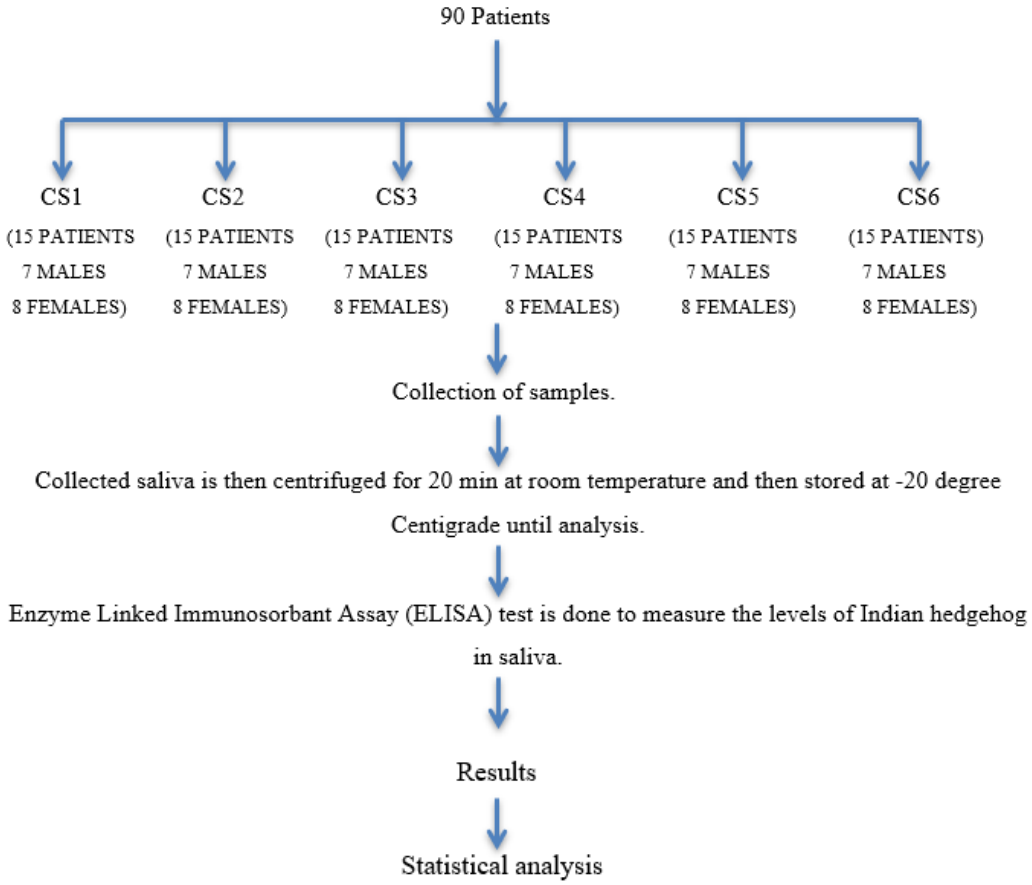
This study is an attempt to correlate salivary levels of Indian hedgehog (Ihh) protein with different stages of cervical vertebrae maturation index (CVMI) in determining the skeletal age.

2. Materials and Methods

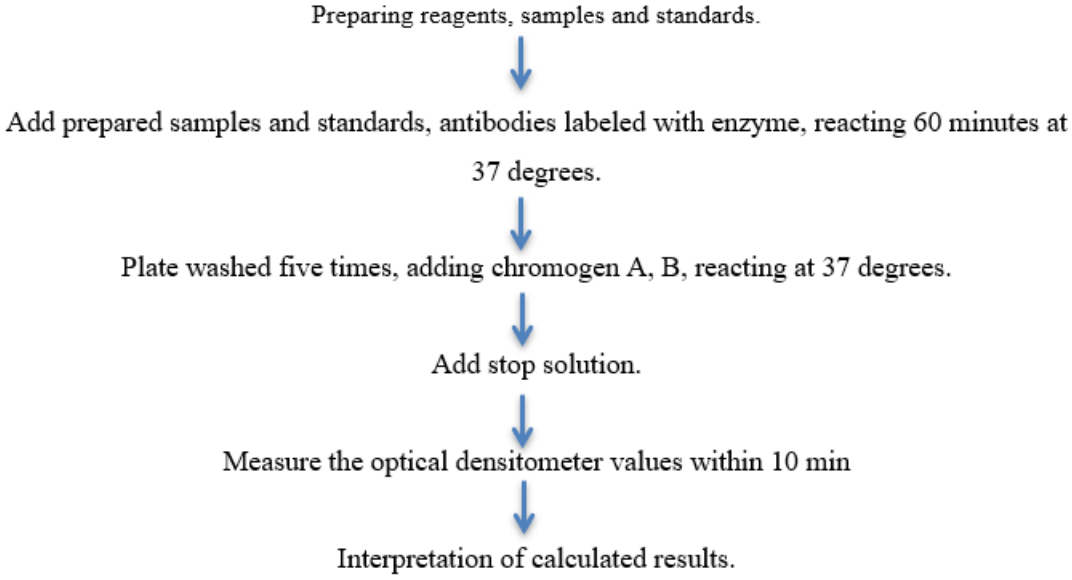
This study was done on patients who reported to Department of Orthodontics and dentofacial orthopedic, and Department of Pedodontics, D.A.P.M.R.V. Dental College and Hospital, Bangalore.

The samples consisted of 90 patients aged 08-20 years (42 males, 48 females) Lateral cephalometric radiographs were obtained and cervical staging was done and evaluated using Hassel and Farman method. 3ml salivary sample was collected from each patient after taking informed consent. The collected saliva was then centrifuged for 20 min at room temperature and then stored at -20°C until analysis. Later Enzyme Linked Immunosorbant Assay (ELISA) test was done to measure the levels of Indian hedgehog in saliva.

Outline of the study



Assay procedure



Inclusion criteria

- Patients seeking orthodontic treatment.
- Patients undergoing orthodontic treatment.
- Post treatment follow- up.
- All patients should be in good general health.

Exclusion criteria

- Patients who had antibiotic therapy within the past 6 months.
- Samples contaminated with blood.
- Patients with systemic illness.

Collection of salivary sample

- The subjects were asked to do mouth wash with chlorohexidine 0.2% solution and refrained from eating for 2 hours and asked to spit into a collection vial until indicated mark (3ml). The collected saliva was then centrifuged for 20 min at room temperature and then stored at -20°C until analysis. The collected salivary samples were subjected to ELISA testing for estimation of Indian hedgehog protein in saliva following established protocols. The results were computed after complete statistical analysis.

Lateral cephalograms

- Lateral cephalometric radiograph of all the ninety subjects were taken using Carestream CS 8000C 73kV/ 12mA/ 13.9 sec.

Evaluation of cervical vertebral maturation stage

- The CVMI technique as described by Hassel and Farman was used to assess the maturational stage of cervical vertebrae. In this technique, the second, third, and fourth cervical vertebrae were examined. The stages of maturation were recorded according to the developmental stages of cervical vertebrae.

Statistical Methods

Shapiro Wilk test was used to check for the normality of data distribution, which revealed that the data was normally distributed. Henceforth, all the inferential analysis was done using relevant parametric tests. One-way Anova test followed by Bonferroni's Post hoc Analysis was used to compare the Mean Salivary Indian Hedgehog levels [ng/ml] between different CVM stages. Independent Student t test was used to compare the difference in the mean Salivary Indian Hedgehog levels [ng/ml] between males & females.

Statistical interpretation

Highly significant $p \leq 0.001$
Significant $p \geq 0.001$ and ≤ 0.05
Not significant $p \geq 0.05$

Statistical Software: Statistical Package for Social Sciences [SPSS] for Windows, Version 22.0. Released in 2013. Armonk, NY: IBM Corp., was used to perform statistical analyses.

3. Results

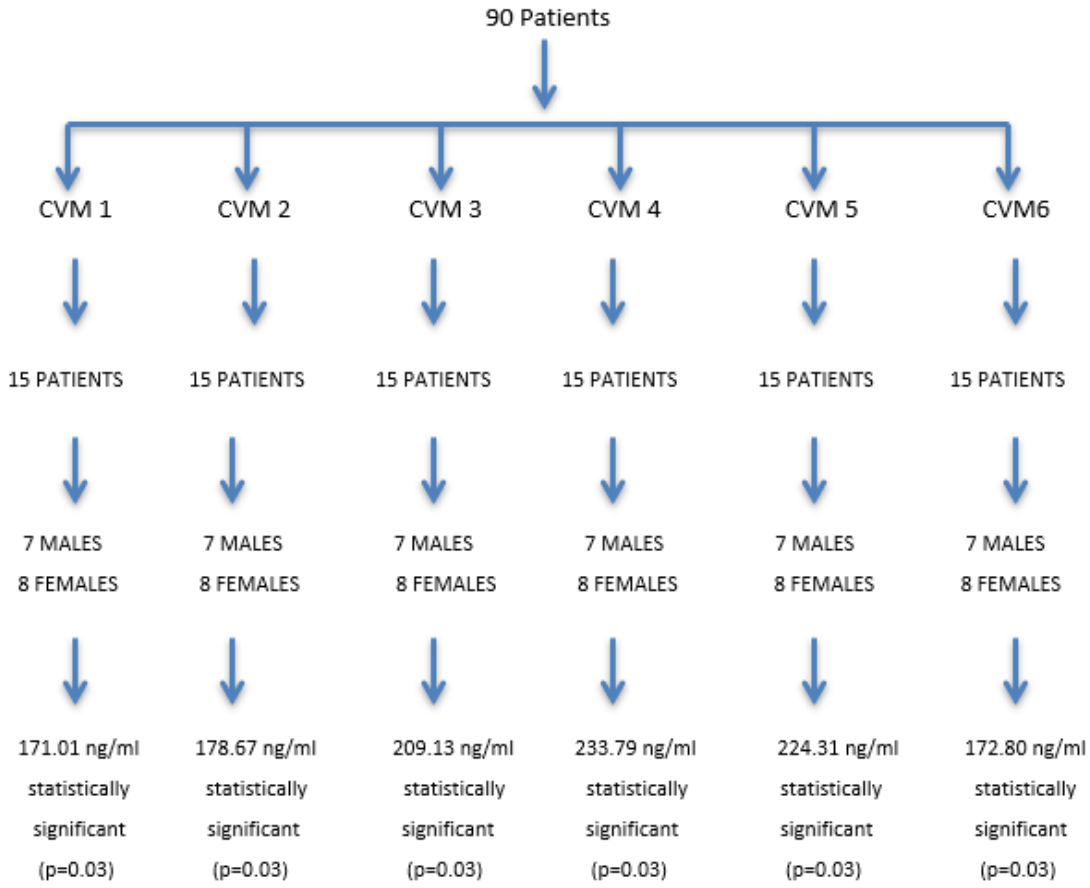
In the present study, the relationship between salivary Indian hedgehog (Ihh) protein levels and cervical maturational stages in 90 subjects were evaluated. Later, the results of these ninety subjects were tabulated according to CVM stages.

Table 1: Indian hedgehog levels in each CVM stages

Sample No.	Cvmi Stage	Gender (M/F)	Age In Years	Result (ng/ml)
1	1	M	8	74.3
2	1	M	8	105.8
3	1	M	8	223.4
4	1	M	8	93.2
5	1	M	8	212.5
6	1	M	8	209.7
7	1	M	8	223.6
8	1	F	8	185.4
9	1	F	8	212.9
10	1	F	8	178.3
11	1	F	8	165.2
12	1	F	8	234.8
13	1	F	8	115.9
14	1	F	8	56.7
15	1	F	8	273.5
16	2	M	8	269.8
17	2	M	9	324.6
18	2	M	9	219.5
19	2	M	8	64.9
20	2	M	10	77.5
21	2	M	9	84.6
22	2	M	10	151.5
23	2	F	9	249.6
24	2	F	9	99.8
25	2	F	9	263.4
26	2	F	8	58.7
27	2	F	9	147.9
28	2	F	9	284.6
29	2	F	8	210.2
30	2	F	9	263.4
31	3	M	11	14F.8
32	3	M	11	273.4

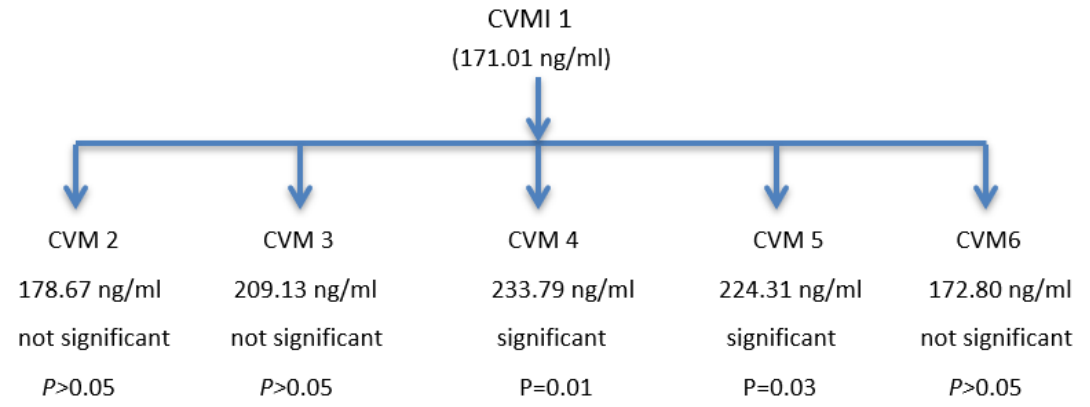
33	3	M	11	349.8
34	3	M	10	208.2
35	3	M	11	78.9
36	3	M	13	234.5
37	3	M	12	69.8
38	3	F	10	283.4
39	3	F	10	165.9
40	3	F	12	212.5
41	3	F	10	201.9
42	3	F	11	275.3
43	3	F	10	220.8
44	3	F	12	249.7
45	3	F	11	265.1
46	4	M	14	164.2
47	4	M	13	223.8
48	4	M	14	215.6
49	4	M	13	239.1
50	4	M	13	219.5
51	4	M	12	152.6
52	4	M	14	167.8
53	4	F	12	303.4
54	4	F	12	174.9
55	4	F	13	212.5
56	4	F	12	261.9
57	4	F	13	254.3
58	4	F	13	367.4
59	4	F	14	192.8
60	4	F	13	214.9
61	5	M	15	178.7
62	5	M	16	225.9
63	5	M	16	169.8
64	5	M	15	155.6
65	5	M	16	84.8
66	5	M	16	132.6
67	5	M	15	224.5
68	5	F	14	109.5
69	5	F	15	242.7
70	5	F	15	59.8
71	5	F	14	103.6
72	5	F	15	157.9
73	5	F	16	302.6
74	5	F	15	215.7
75	5	F	15	228.3
76	6	M	17	203.8
77	6	M	19	279.5
78	6	M	19	265.7
79	6	M	18	248.3
80	6	M	17	215.6
81	6	M	18	152.8
82	6	M	19	115.7
83	6	F	17	248.5
84	6	F	17	231.3
85	6	F	19	262.7
86	6	F	16	284.9
87	6	F	19	153.5
88	6	F	18	272.7
89	6	F	17	322.6
90	6	F	18	249.2

Comparison of Mean Salivary Indian hedgehog levels [ng/ml] between different Cervical Vertebral Maturation stages using One-way ANOVA test.



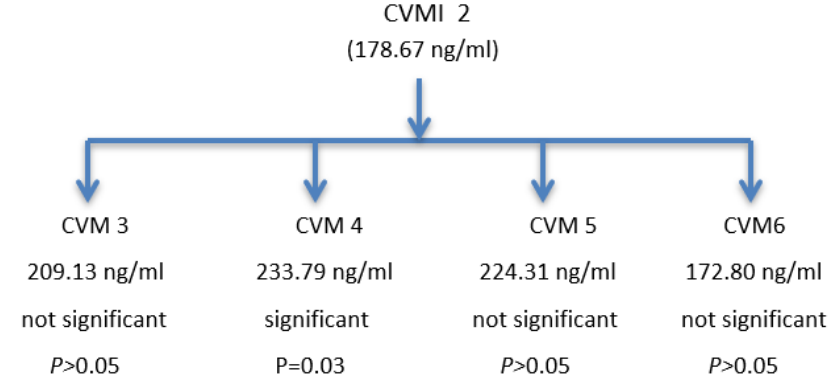
One-way ANOVA test result showed that, there was statistically significant difference (P=0.03) in the comparison of mean salivary Indian hedgehog levels between different Cervical Vertebral Maturation stages (Table 3).

Multiple comparison of Mean Salivary Indian hedgehog levels between different groups using Bonferroni's Post hoc Analysis Test



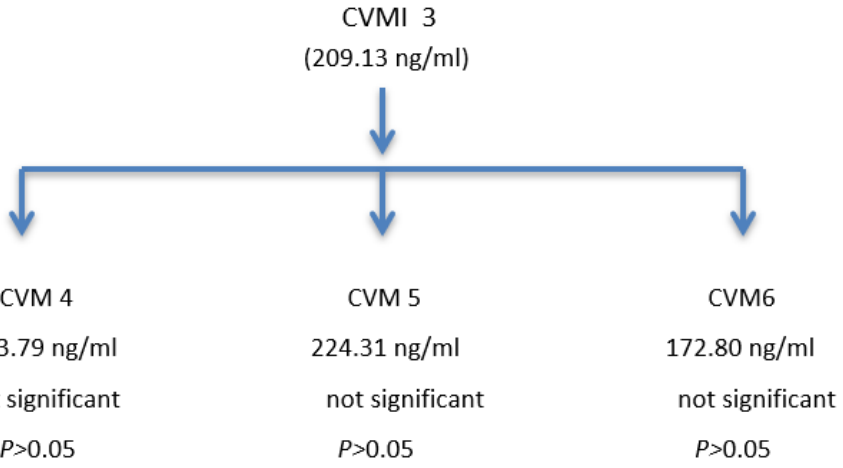
Multiple comparison of mean salivary Indian hedgehog levels between different CVM groups using Bonferroni's Post hoc Analysis Test (Table 4) showed that, there was statistically significant result between

- Stage 1 and stage 4 (P=0.01)
- Stage 1 and stage 5 (P=0.03)



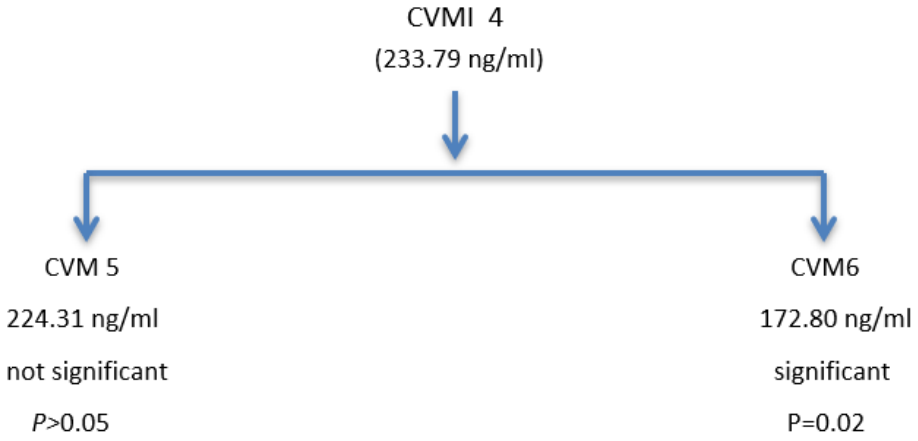
Multiple comparison of mean salivary Indian hedgehog levels between different CVM groups using Bonferroni's Post hoc Analysis Test (Table 4) showed that, there was statistically significant result between

- Stage 2 and stage 4 (P=0.03)



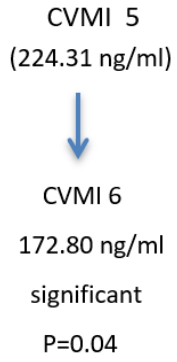
Multiple comparison of mean salivary Indian hedgehog levels between different CVM groups using Bonferroni's Post hoc Analysis Test (Table 4) showed that, there was no statistically significant result between

- Stage 3 and stage 4 (*P*>0.05)
- Stage 3 and stage 5 (*P*>0.05)
- Stage 3 and stage 6 (*P*>0.05)



Multiple comparison of mean salivary Indian hedgehog levels between different CVM groups using Bonferroni's Post hoc Analysis Test (Table 4) showed that, there was statistically significant result between

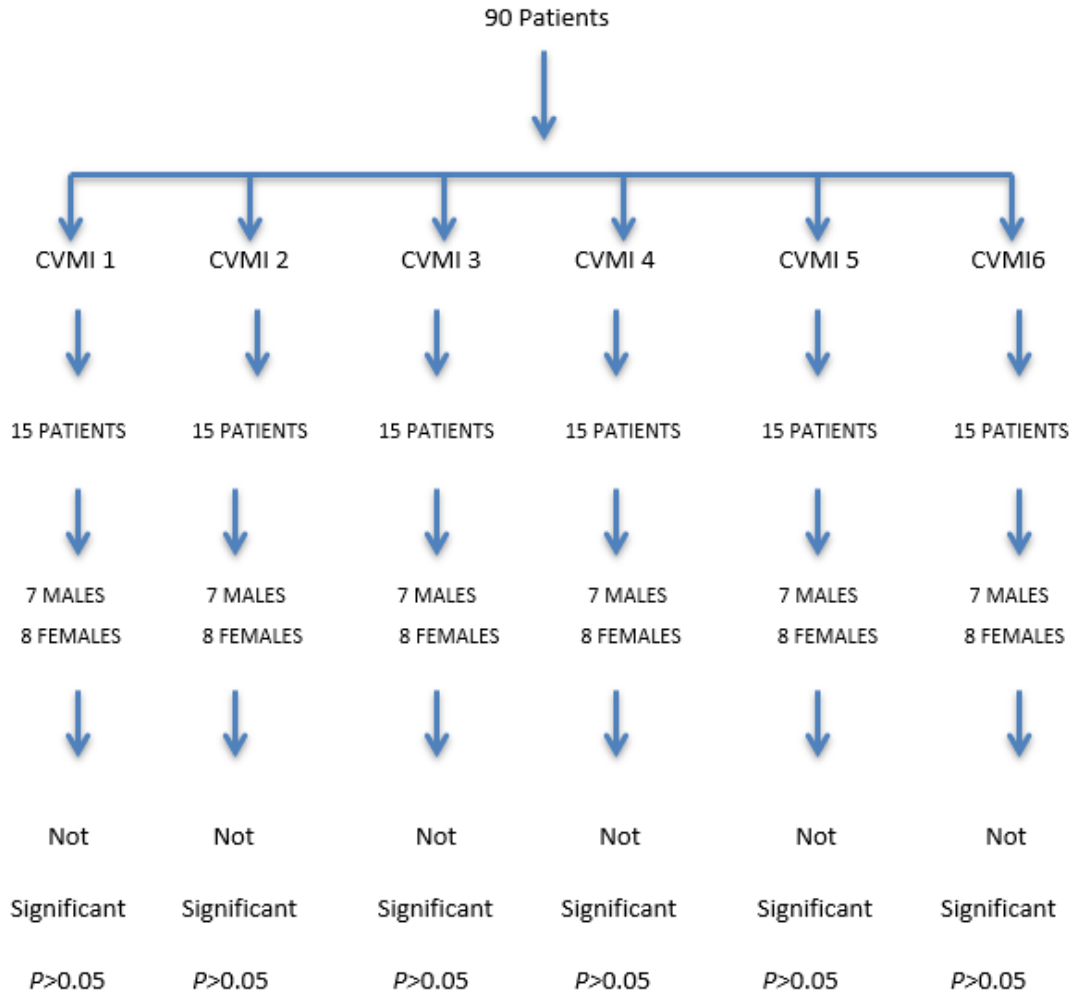
- Stage 4 and stage 6 (P=0.02)



Multiple comparison of mean salivary Indian hedgehog levels between different CVM groups using Bonferroni's Post hoc Analysis Test (Table 4) showed that, there was statistically significant result between

- Stage 5 and stage 6 (P=0.04)

Gender wise comparison of Mean Salivary Indian hedgehog levels [ng/ml] in different stages of Cervical Vertebral Maturation stages using Independent Student test.



Gender wise comparison of mean salivary Indian hedgehog levels in different stages of CVMI stages using Independent Student t-test result showed that, there was no statistically significant difference (P>0.05) between the gender (Table 5).

After statistical analysis

- One-way ANOVA test result showed that, there was statistically significant result (P=0.03) for the comparison of mean salivary Indian hedgehog levels between different Cervical Vertebral Maturation stages (Table 3).
- One-way ANOVA test result showed that, there was a significant rise in the mean salivary Indian hedgehog levels from pre-pubertal to pubertal phase and a gradual decline in the mean salivary Indian hedgehog levels from the pubertal to post pubertal phase (Table 3, Graph 6).
- Multiple comparison of mean salivary Indian hedgehog levels between different CVMI groups using Bonferroni's Post hoc Analysis Test (Table 4) showed that, there was statistically significant result between.
  - Stage 1 and stage 4 (P=0.01)
  - Stage 1 and stage 5 (P=0.03)
  - Stage 2 and stage 4 (P=0.03)
  - Stage 4 and stage 6 (P=0.02)
  - Stage 5 and stage 6 (P=0.04)
- Genderwise comparison of mean salivary Indian hedgehog levels in different CVMI stages using Independent Student t-test result showed that, there was no statistically significant difference (P>0.05) between the gender (Table 5).
- The recommendation of a norm/range for salivary Indian hedgehog levels for each CVM stages was not possible due to overlapping and wide variability in the marker levels which is attributable to limited samples in each of the CVMI stages.
- The present study results can be further validated considering a larger sample size to yield a generalisable norm/range for different CVMI Sstages.

3. Discussion

The degree of skeletal development is a reflection of the level of physiological maturation of a subject. Bone age was shown to be as important as chronological age in evaluating an adolescent's physical development. The assessment of skeletal maturity is an important method in the evaluation, follow up, and timing of therapy in children with growth disorders, such as constitutional growth retardation and growth hormone deficiency [2].

The cervical vertebral maturation as an indicator for skeletal maturity has had its share of proponents and opponents. In a recent study, Perinetti *et al*. concluded that visual assessment of the CVMI stages was accurate and repeatable to a satisfactory level. Although the validity of the CVMI has been questioned occasionally, many authors have proposed its efficacy in assessing growth potential when used with other diagnostic tools [8].

Hassel and Farman stated that since skeletal maturation was a continuous process, one diagnostic tool should not be relied on too heavily. Thus, the various growth maturity indicators must be used together when considering orthodontic corrections to ensure accuracy [9].

The various methods to determine the skeletal maturity are CVMI staging by Baccetti *et al*. Hassel and Farman, hand wrist radiographic method by Fishman, MP3 staging by Hagg

and Taranger, serum IGF-1 by Masoud *et al*. are invasive methods which has either radiation exposure or collection of blood samples [10, 11, 9, 1].

Various systemic and local factors regulate craniofacial growth. Local factors involved in regulation of chondrocyte activity and subsequent endochondral bone growth include Indian hedgehog protein (Ihh), parathyroid hormone related protein (PTHrP), FGF, BMPs, VEGF, SOX 5, 6, 9, RANKL, and OPG [2]. Indian hedgehog protein has been reported to regulate multiple steps in the development of growth plate and the secondary cartilage at the head of the condyle during skeletal morphogenesis [6].

Biomarkers have the advantage of avoiding invasive x- ray exposure. They can be measured from various biologic fluids such as blood, saliva, and urine, thereby overcoming the subjectivity associated with radiographs [4].

Blood specimens are used extensively to monitor the general state of health and for analysis of many specific diagnostic analytes. Drawing blood can be impractical for people with blood- injection-injury type phobia and those who require daily monitoring of biomarker levels [12].

Non-invasive technology has thus become increasingly important and would be ideal for point-of-care diagnosis.[12] Saliva is one of the most preferable and practical specimens for health monitoring, as it is readily available as well as easily collected and stored [15]. The advantages of saliva over blood samples are lower overall cost, lower infection risk, increased patient convenience, acceptability, compliance and uptake [13].

Saliva can be collected in large amounts in a noninvasive manner. The discovery of salivary biomarkers holds potential for early prediction of skeletal maturation and useful in making diagnostic choices in orthodontics.

Determination of maturation and subsequent evaluation of growth potential are extremely important. Our goal was to evaluate the validity of salivary Indian hedgehog levels in different cervical maturational stages to assess the skeletal maturation.

Table 1 shows the Indian hedgehog levels (ng/ml) in each CVMI stages for males and females, sample number and their respective age in years.

Table 2 and graph 1 shows the age distribution of the samples in each CVMI stage. The mean age for stage 1 was 8 years, stage 2 was 8.9 +/- 0.6 years, stage 3 was 11 +/- 0.9 years, stage 4 was 13 +/- 0.8 years, stage 5 was 15.2 +/- 0.7 years, stage 6 was 17.9 +/- 1 years.

Table 3 and graph 2 shows the gender distribution of each stage. The study sample consists of 7 males and 8 females for each CVMI stage, which comprises a total of 90 samples (42 males and 48 females).

Table 4 shows the Comparison of Mean Salivary Indian Hedgehog levels [ng/ml] between different Cervical Vertebral Maturation stages using One-way ANOVA test. The mean salivary Indian hedgehog level in CVMI stage 1 was 171.01 ng/ml, stage 2 was 178.67 ng/ml, stage 3 was 209.13 ng/ml, stage 4 was 233.79 ng/ml, stage 5 was 224.31 ng/ml and stage 6 was 172.80 ng/ml.

Graph 4, 5 shows that there was a gradual increase in the mean salivary Indian hedgehog level from CVM stage 1 to stage 4 where the mean Indian hedge level reaches its peak in CVMI stage 4 and there was a decrease in mean salivary Indian hedgehog level from CVMI stage 4 to stage 6.

This result was similar to the results found by Sinha [4] *et al*. in 2016 where the mean serum and urine IGF-1 levels increases from CVMI stage 1 to stage 4 and decreases from CVMI stage 4 to stage 6. The mean serum and urine IGF-1 level reaches its peak in CVMI stage 4.

Table 5, shows the result of multiple comparison of mean salivary Indian hedgehog levels between different groups using Bonferroni's Post hoc Analysis Test.

Table 6 and graph 5, shows the Gender wise comparison of mean salivary Indian hedgehog levels [ng/ml] in different stages of Cervical Vertebral Maturation stages using Independent Student test. The mean salivary Indian hedgehog level for males and females in CVM stage 1 was 163.21 ng/ml and 177.84 ng/ml, stage 2 was 157.49 ng/ml and 197.20 ng/ml, stage 3 was 180.34 ng/ml and 234.33, stage 4 was 211.63 ng/ml and 253.18 ng/ml, stage 5 was 197.51 ng/ml and 247.56 ng/ml, and stage 6 was 167.41 ng/ml and 177.51 ng/ml respectively.

Graph 6 shows the mean salivary Indian hedgehog levels in different pubertal phases. The result shows that there was an increase in the mean salivary Indian hedgehog levels from pre pubertal phase to pubertal phase and it decreases from pubertal to post pubertal phase. The mean salivary Indian hedgehog level reaches its maximum in the pubertal phase.

This result was similar to the result found by Gupta [14] *et al*. in 2012 where the mean serum IGF-1 level increases from pre pubertal to pubertal phase and decreases from pubertal to post pubertal phase.

The results of the present study could not be compared directly with any other studies, as this was the first study to be conducted on saliva for Ihh estimation.

Although obtaining salivary samples is not a common practice in orthodontics, we encountered less resistance in collecting them than we had expected. Making the subjects aware of the interdependence among growth, orthodontic treatment options, and the stability of treatment results followed by an explanation of the simplicity of the procedures involved, we saw that the subjects were more than willing to participate in our study and know the results.

The present study results can be further validated considering a larger sample size to yield a generalizable norm/range for different CVMI stages.

More research is necessary to validate these results in a different population and with a larger sample. We propose longitudinal studies to confirm the utility of salivary Ihh, in accurately determining the timing and possibly the intensity of a patient's growth spurt and to determine whether salivary Ihh is a good predictor of skeletal maturity.

Table 2: Age distribution of the sample

Age distribution of the sample		
Group	**Mean**	**SD**
Stage 1	8.0	0.0
Stage 2	8.9	0.6
Stage 3	11.0	0.9
Stage 4	13.0	0.8
Stage 5	15.2	0.7
Stage 6	17.9	1.0

Table 3: Gender distribution of the sample

Sex distribution of the sample		
Group	**Males**	**Females**
Stage 1	7	8
Stage 2	7	8
Stage 3	7	8
Stage 4	7	8
Stage 5	7	8
Stage 6	7	8

Table 4: Comparison of Mean Salivary Indian hedgehog levels [ng/ml] between different Cervical Vertebral Maturation stages using One-way ANOVA test

CVM	N	Mean	SD	Std. Error	Min	Max	F	P-Value
Stage 1	15	171.01	66.07	17.06	56.7	273.5	2.563	0.03**
Stage 2	15	178.67	84.11	21.72	58.7	284.6		
Stage 3	15	209.13	67.24	17.36	69.8	283.4		
Stage 4	15	233.79	56.57	14.61	115.7	322.6		
Stage 5	15	224.31	56.68	14.63	152.6	367.4		
Stage 6	15	172.80	67.42	17.41	59.8	302.6		

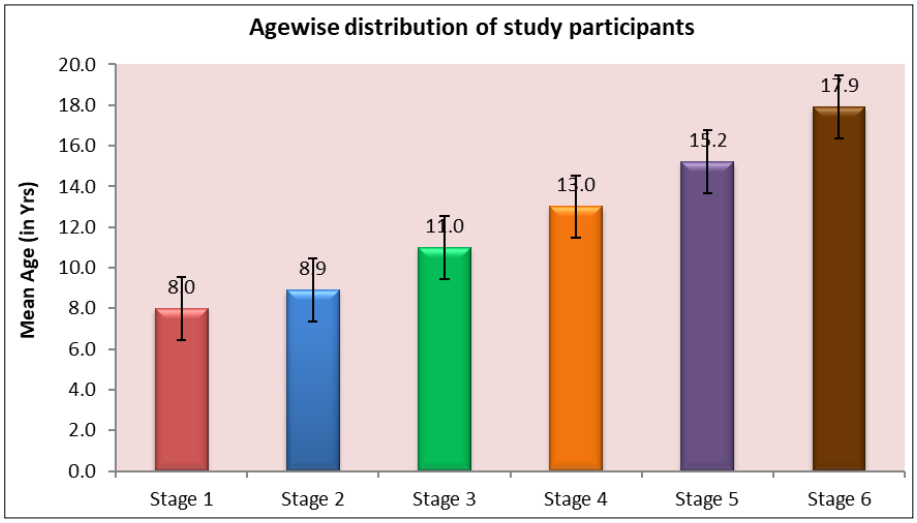
Highly significant $p<0.001$***
Significant $p<0.05$ **
Not significant $P>0.05$*

Table 5: Multiple comparison of Mean Salivary Indian hedgehog levels between different groups using Bonferroni's Post hoc Analysis Test

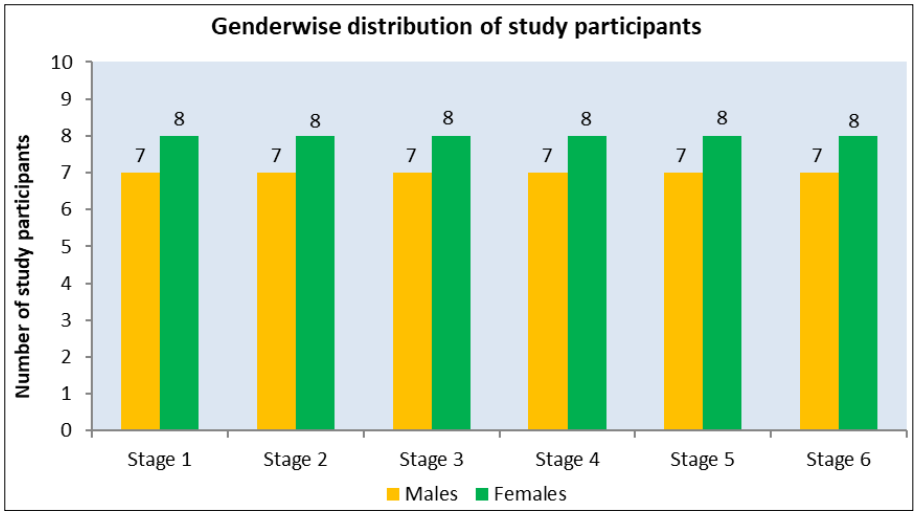
Group [I]	Group [J]	Mean Diff.	95% CI for the Diff		P-Value
			Lower	Upper	
Stage 1 (171.01)	Stage 2 (178.67)	-7.65	-56.29	40.98	0.76
	Stage 3 (209.13)	-38.12	-86.76	10.52	0.12
	Stage 4 (233.79)	-62.77	-111.41	-14.14	0.01***
	Stage 5 (224.31)	-53.30	-101.94	-4.66	0.03**
	Stage 6 (172.80)	-1.79	-50.42	46.85	0.94
Stage 2 (178.67)	Stage 3 (209.13)	-30.47	-79.10	18.17	0.22
	Stage 4 (233.79)	-55.12	-103.76	-6.48	0.03**
	Stage 5 (224.31)	-45.65	-94.28	2.99	0.07
	Stage 6 (172.80)	5.87	-42.77	54.50	0.81
Stage 3 (209.13)	Stage 4 (233.79)	-24.65	0.32	-73.29	23.98
	Stage 5 (224.31)	-15.18	0.54	-63.82	33.46
	Stage 6 (172.80)	36.33	0.14	-12.30	84.97
Stage 4 (233.79)	Stage 5 (224.31)	9.47	0.70	58.11	39.16
	Stage 6 (172.80)	60.99	0.02**	12.35	109.62
Stage 5 (224.31)	Stage 6 (172.80)	51.51	0.04**	2.88	100.15

Table 6: Gender wise comparison of Mean Salivary Indian hedgehog levels [ng/ml] in different stages of Cervical Vertebral Maturation stages using Independent Student test

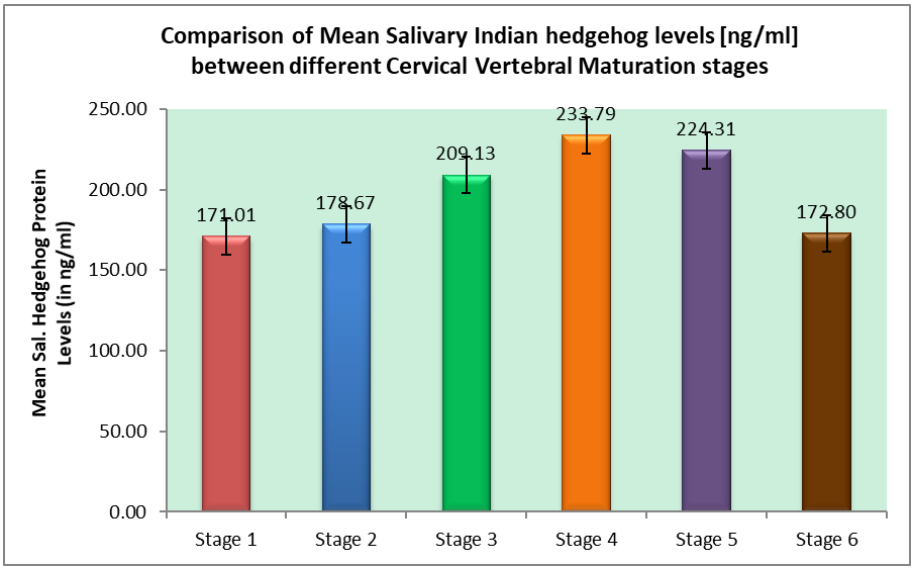
CVM	Gender	N	Mean	SD	S.E.M	Mean Diff	t	P-Value
Stage 1	Males	7	163.21	68.27	25.80	-14.62	-0.415	0.69
	Females	8	177.84	67.98	24.04			
Stage 2	Males	7	157.49	84.38	31.89	-39.71	-0.907	0.38
	Females	8	197.20	84.87	30.01			
Stage 3	Males	7	180.34	82.42	31.15	-53.98	-1.642	0.12
	Females	8	234.33	40.82	14.43			
Stage 4	Males	7	211.63	60.02	22.69	-41.55	-1.478	0.16
	Females	8	253.18	48.92	17.29			
Stage 5	Males	7	197.51	34.74	13.13	-50.25	-1.857	0.09
	Females	8	247.76	63.59	22.48			
Stage 6	Males	7	167.41	49.98	18.89	-10.10	-0.280	0.78
	Females	8	177.51	83.03	29.36			



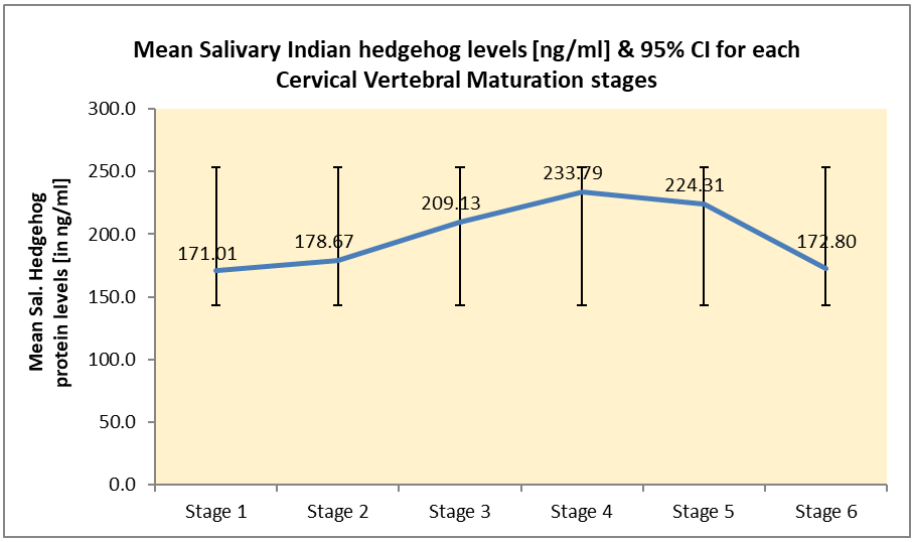
Graph 1: Age wise distribution of study participants



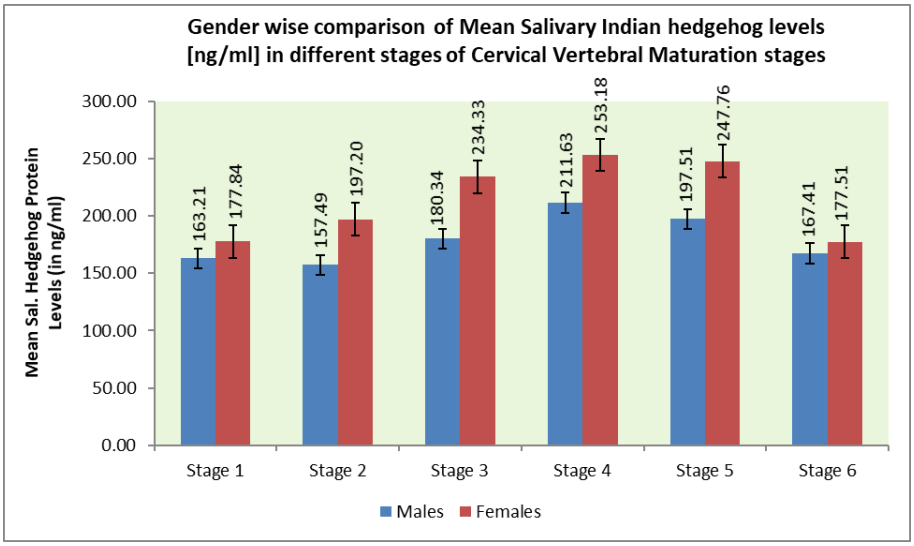
Graph 2: Gender wise distribution of study participants



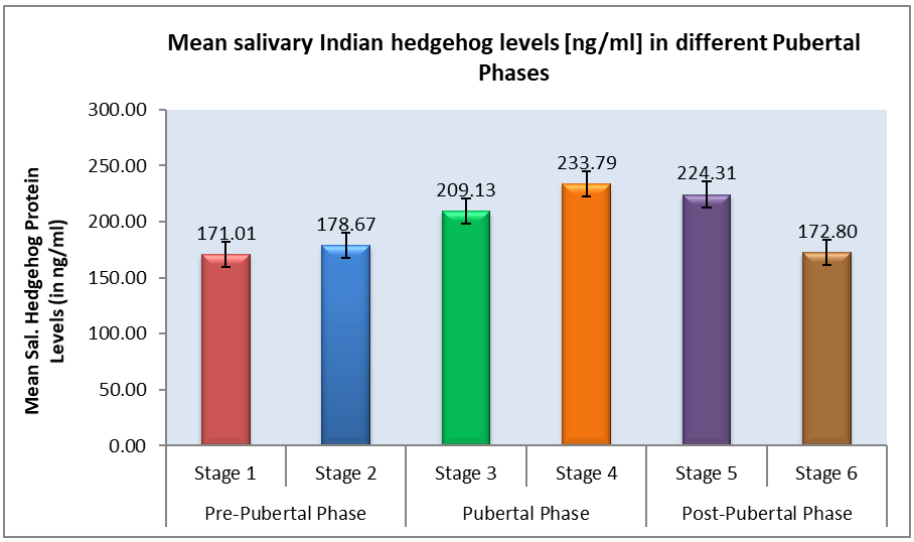
Graph 3: Comparison of mean Salivary Indian hedgehog levels [ng/ml] between different Cervical Vertebral Maturation stages



Graph 4: Mean salivary Indian hedgehog levels [ng/ml] & 95% CI for each Cervical Vertebral Maturation stages



Graph 5: Gender wise comparison of mean salivary Indian hedgehog levels [ng/ml] in different stages of Cervical Vertebral Maturation stages



Graph 6: Mean salivary Indian hedgehog levels [ng/ml] in different Pubertal Phase

4. Conclusion

The conclusions drawn from this study are:-

1. This study indicates that there is a significant association between mean salivary Indian hedgehog levels and cervical vertebral maturational stages.
2. There is a significant association between mean salivary Indian hedgehog levels at different pubertal phases.
3. There is no significant association between gender and mean salivary Indian hedgehog levels in different cervical maturational stages.
4. The findings of the study suggest that salivary Indian hedgehog can be used as a biomarker to measure the skeletal maturity of an individual.

5. References

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